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13. ABSTRACT *(Maximum 200)*

In this report we investigate the role of ECM proteins and growth factors in anchorage-independent survival and growth of a murine mammary carcinoma-derived cell line, designated SP1. We demonstrate that SP1 cells adhere well to FN and VN and express a number of integrins, including $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_6\beta_1$, $\alpha_3\beta_1$ and $\alpha_2\beta_1$, the latter being upregulated under anchorage-independent conditions. We also demonstrated by partially removing FN from 7% FBS or adding FN to 1% FBS that FN is required for SP1 cell colony growth.

Cl-12-H, an SP1 cell clone with efficient colony forming ability, was more adherent to Coll I and expressed higher levels of $\alpha_2\beta_1$ than SP1 cells or the clone. FN and Coll I were both able to promote colony growth of Cl-12-H cells, but had no effect on Cl-24-L cells.

We also demonstrated that FN and HGF together, acted in a additive fashion on cell survival. Using either wortmannin or Cl-12-H transfected with a dominant negative mutant p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) that the additive survival effect mediated by FN+HGF together was very dependent on PI 3-kinase.

HGF receptor phosphorylation was reduced under anchorage-independent conditions but showed increased tyrosine phosphorylation in response to exogenous HGF. PI 3-kinase showed reduced phosphorylation under anchorage-independent conditions and this tyrosine phosphorylation was not increased in response to FN or HGF. In contrast, PI 3-kinase activity was increased in a stepwise manner in the presence of FN, HGF and FN + HGF. Together these results demonstrate that ECM-integrin interactions and growth factors are involved in anchorage-independent survival and growth of SP1 cells.

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Introduction

Breast cancer is the most common cancer among North American women. It is the number two cause of death (after lung cancer) of women ages 35-54 and affects approximately 1 in 9 women in their lifetime; of these one third will die of metastases. Unlike lung and skin cancer, the causes of breast cancer are not well understood. Conventional treatment modalities such as surgery, chemotherapy and radiation, whether used alone or in combination are not always effective at destroying all of the metastatic lesions. Better or different treatments are now required to combat breast cancer. These new treatments can be obtained through a better understanding of tumor cell biology. An important characteristic of tumor cells, with relevance to prognosis, is their ability to metastasize and grow anchorage-independently.

Anchorage-independent growth

Non-transformed cells must be adherent and spread in order to proliferate. As the cells progressively become transformed there is a progressive loss of anchorage requirement and response to exogenous growth factors via paracrine stimulation. The term anchorage-independent growth refers to growth and proliferation in the absence of adhesion and spreading. The classical assay to determine the anchorage-independent potential of cells is growth in soft agar. Cells which are able to grow in soft agar generally are more tumorigenic in mice (Shin *et al.*, 1975). These properties suggest a relationship between the degree of transformation and the anchorage-independent growth potential. The term anchorage-independence was coined many years ago before the understanding of cell adhesion and integrins. We, in this report, and others (Glimelius *et al.*, 1988; Nederman *et al.*, 1984) have shown the involvement of integrins and extracellular matrix (ECM) in cells which are not adherent or spread, which conflicts with the original term anchorage-independence. From herein, the term "anchorage-independence" will refer to spreading independence or growth in a three dimensional matrix. In our survival assay the cells are maintained in suspension in media while they are imbedded in a three-dimensional agar matrix in the colony assays.

Tumor nodules have been shown to be more resistant to chemotherapy and radiation therapy than adherent

cells. A tumor spheroid typically shows several layers of cells with a necrotic center, several layers of cells undergoing apoptosis, few layers of quiescent cells and an outer layer of rapidly proliferating cells (Olive and Durand, 1994). As the spheroids enlarge, the external well-nourished cells continue to divide but the internal cells, lacking nutrients and O_2 often exit the cell cycle and enter G_0. It is these cells which have exited the cell cycle which are likely to be resistant to chemotherapy and radiotherapy since these treatments are designed for rapidly dividing cells.

No correlation was found in a survey of the anchorage-independent growth of epidermal keratinocyte and human squamous cell carcinoma cell lines and oncogene expression (Kim *et al.*, 1991). In addition transformation of rat embryo fibroblasts with v-Src transformed 95% of the cells but only 25% were anchorage-independent (Tavoloni *et al.*, 1994). Despite these studies, oncogenic transformation can render some cells anchorage-independent (Masuda *et al.*, 1992; Rak *et al.*, 1995). These results suggest that extracellular factors, in addition to growth factors, may have a contributing factor in anchorage-independent growth.

Integrins and ECM

The ECM is composed primarily of collagens, glycoproteins and proteoglycans. ECM is not just the glue between cells as previously thought, but provides the scaffolding, support and strength to tissues and organs. In addition, almost all cellular events occurring during embryonic development and organogenesis, tissue remodeling, wound healing or tumor growth and metastasis are in some way influenced by individual components of the ECM. There are three mechanisms by which ECM can regulate cell behavior; (1) composition of ECM, (2) regulation of cell surface receptors that mediate adhesion to ECM, and (3) synergistic interactions between growth factors and ECM (Adams and Watt, 1990). These mechanisms are not mutually exclusive and all three can occur simultaneously.

Integrins are transmembrane glycoprotein heterodimers composed of an α and β subunit noncovalently bound together which mediate cell-cell and cell-matrix interactions. Associations of the α and β subunits are not random, although α_v associates with multiple β subunits, most α subunits associate with only one β subunit (Hynes, 1987, Albelda and Buck, 1990).

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Integrins play a key role in a number of biological processes where such as embryogenesis, wound healing, cell differentiation, lymphocyte migration, survival, cell-cell interactions, cell migration and gene transcription (reviewed in Ruoslahti, 1991; Giancotti and Mainiero, 1994). Integrins are also involved in a number of pathological processes such as bacterial cell invasion (Isberg and Leong, 1990) and tumor cell migration and invasion (Dedhar, 1990; Dedhar and Saulnier, 1990).

Integrins and growth factors in anchorage-independent growth

There is limited information on the role of ECM proteins and integrins in anchorage-independent growth of either normal or transformed cells. The presence of ECM protein such as fibronectin (FN), laminin (LM), collagen and proteoglycans in tumor spheroids has been demonstrated many years ago in glioma cell lines (Glimelius *et al.*, 1988; Nederman *et al.*, 1984; De Pauw-Gillet *et al.*, 1988). More recently, bovine granuloma cells cultured in agar produced FN and collagen type IV (Coll IV) (Rodgers *et al.*, 1995).

There were several studies performed on the expression of integrins in tumor spheroids. The α_6 and β_1 subunits were decreased in tumor spheroids of squamous cells A431, while there were no changes in the α_2, α_5, β_4 (Waleh *et al.*, 1994). Hauptmann *et al.*, (1995) investigated the expression of integrins on colorectal cells growing either as monolayers or spheroids *in vitro*, or as tumors and *in vivo* tumors in nude mice. They showed that different integrins were either decreased or increased in the different culture conditions and concluded that the different microenvironments regulated integrin expression. In addition, fibroblasts typically downregulated both FN and $\alpha_5\beta_1$ expression when maintained in suspension (Dalton *et al.*, 1992).

Growth factors are capable of driving adherent cells through the cell cycle. However, untransformed cells such as 3T3 L1 or NRK fibroblasts will not progress through the cell cycle, in the presence of growth factors, if maintained in suspension. Transformed cells have acquired growth factor independence through autocrine secretion of growth factors, mutations or unregulated expression in growth factor receptors or proteins involved in signal transduction, both of which cause constitutive activation. The role of several growth factors in anchorage-independent growth has been investigated. Growth factors such as EGF, FGF and PDGF can increase anchorage-independent growth of some cells in the presence of 10% serum (Rizzino *et al.*, 1986). TGF-

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β is well documented in the literature as being able to increase anchorage-independent growth of a number of cell lines and affects the expression of both ECM proteins such as FN, as well as their corresponding integrin receptors (Heino et al., 1989; Roberts et al., 1992). It is not known what effect TGF-β has on ECM and integrin expression on anchorage-independent cells.

A recent development is that integrin-mediated adhesion and signaling is required for survival. Detachment from a substrate causes the cells to undergo apoptosis. This mechanism for apoptosis has been termed anoikis (Frisch and Francis, 1994). Only integrin-mediated attachment circumvents anoikis; attachment to antibodies directed to MHC proteins or plated on a polylysine substrate, failed to rescue the cells from apoptotic death.

Integrin and growth factor signal transduction

There is overlap between receptor tyrosine kinase and integrin signal transduction pathways. It appears that there may be additional, adhesion specific events involved in mitogenesis, because high concentrations of growth factors are not sufficient to override the adhesion requirement (Kang and Krauss, 1996). However, the molecular mechanisms which join integrin and growth factor signalling pathways are not well understood. There are a number of ways in which ECM and growth factors act co-operatively. (1) The ECM presents bound growth factors to cells in a functional manner (2) the ECM may change the cell shape through adhesive interactions and render it responsive to growth factors (3) ECM proteins may bind to integrins and generate signals that interact with those generated by growth factors (Schubert, 1992). One function of the ECM which is important for growth factor signalling is the activation of PIP 5-kinase catalyzed PIP_2 formation by adhesion to FN which is ready to be used in growth factor signalling pathways (McNamee et al., 1993).

There are a number of signalling molecules which are well established in the growth factor signalling pathways which are now being demonstrated to be involved in integrin signalling pathways. For example Ras, Grb2, Src and FAK have been implicated in such signalling pathways. These results suggest cooperative or synergistic interactions between growth factors and integrin signal transduction pathways. Until recently the effect of adhesion via integrins and the effects of growth factor on cellular proliferation and other functions were

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studied independently. Most studies performed on the cooperative effect between integrins and growth factors have been done in fibroblasts and platelets. Little information is available in this area on epithelial cells.

Cell survival and anchorage

It has been shown that anchorage-dependent cells when denied attachment, not only stop proliferating but also undergo apoptosis (Meredith *et al.*, 1993; Frisch and Francis, 1994). How anchorage-independence relates to integrin signalling and what role $\alpha_5\beta_1$ might play in anchorage-independent growth is yet to be resolved. CHO cells transfected with the $\alpha_5\beta_1$ integrin were able to inhibit serum deprived apoptosis on immobilized fibronectin by upregulating Bcl-2 but those transfected with $\alpha_v\beta_1$ could not inhibit apoptosis (Zhang *et al.*, 1995). It was also found that the α_5 cytoplasmic domain was required for survival on fibronectin. Montgomery *et al.*, (1994) have shown that ligation of the vitronectin (VN) receptor $\alpha_v\beta_3$ within a three-dimensional dermal collagen matrix, suppresses apoptosis and promotes melanoma (M21) cell growth. CHO and osteosarcoma cells cultured in serum-free conditions, survived only if they attached through the $\alpha_5\beta_1$ integrin. Whereas some other integrins, while supporting cell attachment, could not rescue the cells from apoptosis (Zhang *et al.*, 1995). FAK has also been implicated in the maintenance of survival since FAK knockouts in cell lines were undergoing apoptosis (Frisch *et al.*, 1996).

Extracellular matrix proteins and growth factors are key regulators of cell survival and growth under adherent conditions. Cells deprived of serum or maintained under anchorage-independent conditions often undergo cell death via apoptosis (Frisch and Francis, 1994). Adhesion to ECM proteins via integrins has been shown to mediate survival of adherent cells (Ruoslahti and Reed, 1994; Zhang *et al.*, 1995). Basement membrane proteins, provided by Matrigel, were required for suppression of apoptosis in mammary epithelial cells (Boudreau *et al.*, 1995, 1996). Adhesion to FN via $\alpha_5\beta_1$ was required for survival of CHO cells in serum free-conditions (Zhang *et al.*, 1995). These results provide evidence that occupancy of integrin receptors provides a cell survival signal.

A number of growth factors, including epidermal growth factor (EGF), platelet derived growth factor (PDGF), and nerve growth factor (NGF) can also promote cell survival by a PI 3-kinase-dependent pathway (Yao and Cooper, 1995, 1996). In addition, we have previously shown that PI 3-kinase is required for the

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hepatocyte growth factor (HGF)-mediated mitogenic response on adherent SP1 cells (Rahimi *et al.*, 1996b).

Very little is known about the role of ECM proteins and growth factors in tumor cell survival under anchorage-independent conditions. It is known that soluble FN can bind to the surface of fibroblasts and hepatocytes in suspension (Akiyama and Yamada, 1985; Johannson, 1985). Soluble VN was not sufficient to inhibit apoptosis in normal endothelial cells (Re *et al.*, 1994). Although some tumor cells have bypassed the requirement for adhesion, it is not known whether ECM proteins still function in providing a growth or survival signal when under anchorage-independent conditions.

In conclusion ECM-integrin interactions and cooperative signalling with growth factors play an essential role in both normal and transformed cell behaviour. The interactions have been well studied in adherent cells but are not well understood in three-dimensional spheroids, which is an intermediate model between cells in culture and *in vivo* tumor nodules.

Rationale and Hypothesis

Tumor spheroids have been used as a tumor model for a number of years. They were shown to histologically resemble *in vivo* tumors and are more physiologically similar to *in vivo* tumors than cells cultured in monolayers. Tumor spheroids are an intermediate system between tissue culture and *in vivo* tumors. Roskelley and Bissell (1995) found that mammary cells not only required basement membrane proteins to differentiate but also required a rounded morphology. Howlett *et al.* (1995) have shown that normal mammary cells differentiate into well-organized acinar structures whereas tumor cells form large, disorganized colonies when plated in the basement membrane matrix, Matrigel.

Acquiring a better understanding of how extracellular factors such as ECM and growth factors affect metastatic cell growth in an anchorage-independent manner as found in tumor spheroids may provide the basis for developing better therapies to treat metastatic lesions.

The mammary gland is a good system to investigate epithelial cell proliferation, differentiation and tumorigenesis. Unlike other organs, the mammary gland develops to maturity in the adult animal, reaching its fully functional status only during late stages of pregnancy and lactation. Pregnancy induces a massive proliferation of

the epithelial cells, which leads to a branching morphogenesis which is accompanied by the production and secretion of milk proteins by the epithelial cells. After the lactation period, the epithelium is dismantled during involution by a process that involves degradation of the ECM prior to apoptosis, suggesting that the ECM is required for survival of mammary epithelial cells (Pullan *et al.*, 1996).

SP1 cells are a heterogenous population of tumor cells, therefore individual clones may have different adhesive and growth properties. The unstable genotype of tumor cells may cause some drift in the phenotypes in a rapidly growing population of cells. It is difficult to properly assess the adhesive and integrin properties in a heterogenous population of cells. As seen above, ECM proteins have both positive and negative influences on colony growth of SP1 cells. It is possible that some of the cells within the heterogenous population of SP1 cells respond positively to ECM proteins while others respond negatively. Such a situation may result in a null effect of the ECM or inconsistent results between experiments. It should therefore be possible to isolate a population of SP1 cells which grow efficiently in agar and another population which does not grow in agar.

We have previously demonstrated that mammary fat tissue or 3T3-L1 preadipocyte conditioned media can stimulate the growth of a murine mammary carcinoma cell line, SP1 (Elliott *et al.*, 1988, 1992; Rahimi *et al.*, 1996a). These cells grown in soft agar contain abundant extracellular FN in the form of fibrils. Much of what is known about the effects of ECM on mammary cells is based on differentiation and not growth. Much of what is known about cell growth under anchorage-independent conditions is based on growth factors and normal fibroblasts and concentrates on the cell cycle. *There are few studies on the role of ECM in anchorage-independent survival and growth of tumor cells.* This thesis will look primarily at the effects of ECM on anchorage-independent growth but also considers growth factors. We now want to show how extracellular matrix and growth factors participate in anchorage-independent survival and growth of SP1 cells. **Our hypothesis is that extracellular matrix proteins and growth factors stimulate their respective cell surface receptors under anchorage-independent conditions and generate intracellular signals which permit survival and colony growth of SP1 cells.**

1. Our first objective was to characterized the integrins and extacellular matrix proteins involved in anchorage-independent growth of SP1 cells.

A) Adhesive and integrin profiles of SP1 cells

SP1 cells are an adherent murine mammary carcinoma cell line. They adhered and spread extensively to fibronectin and vitronectin over a wide range of concentrations (0.16-20 μg/ml). They adhered moderately well to collagen type I, and show a concentration dependent requirement of approximately 1.5 μg/ml to collagen type IV. SP1 cells attached poorly to laminin and few cells remained on BSA coated plates after washing with phosphate buffered saline (PBS) (Figure 1). We observed a positive correlation between the degree of spreading and the strength of adhesion of SP1 cells. SP1 cells adherent on FN and VN show a more flattened morphology as compared to Collagen type I and Laminin, onto which they spread poorly and show a spindle-shaped morphology (Figure 2)

Cell adhesion to the ECM can regulate integrin expression (Chen *et al.*, 1992; Dalton *et al.*, 1992), therefore we determined the integrin profiles of SP1 cells cultured on plastic or anchorage-independently for 24 h in the presence of 7% FBS. We have used a number of different antibodies to immunoprecipitate ^{125}I-labelled integrins from the cell surface of SP1 cells. Integrin α subunits are typically 120-150 kDa, with the exception of α_1 subunit which is 180 kDa. The β_1 subunit is 110 kDa and the β_3 subunit is 97 kDa. Immunoprecipitations using antibodies directed to one subunit will often co-precipitate the associated subunit(s). For example in the anti-β_1 immunoprecipitation in Figure 3, the antibody is directed to the β_1 subunit and will co-precipitate all associated α subunits. Therefore the upper band, which is difficult to distinguish from the lower band in this exposure, is composed of numerous α subunits, and the lower band of the β_1 subunit. In the immunoprecipitation using antibodies specific for α_2, α_3, α_5, α_6, or α_v subunits, co-precipitation of the corresponding β subunit is also observed. The association between some α and β subunits is not strong, and may be lost when using a strong lysis buffer such as RIPA. For example, there is only a weak β band associated with the α_v, α_2 and α_6 subunit. SP1 cells express a number of integrins on the cell surface including the FN receptor, $\alpha_5\beta_1$, the VN receptor, $\alpha_v\beta_3$, the LM receptor, $\alpha_6\beta_1$ and the CollI, LM and FN receptor, $\alpha_3\beta_1$. The collagen/LM receptor, $\alpha_2\beta_1$, was weakly expressed on SP1 cells

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cultured on plastic but was upregulated on SP1 cells which were maintained under anchorage-independent conditions for 24 h (Figure 3). Flow cytometry revealed very low expression of the FN receptor $\alpha_4\beta_1$ (data not shown). These data show that SP1 cells adhere to a number of ECM proteins and express a number of different integrins on the cell surface.

Integrin receptors require divalent cations such as Ca^{2+}, Mg^{2+}, or Mn^{2+} for adhesion. To provide further evidence that integrin receptors are the primary receptors for extracellular matrix proteins on SP1 cells we investigated the role of divalent cations on SP1 cell adhesion to fibronectin. We removed the divalent cations from the cell surface by incubating the cells in divalent cation-free PBS containing 5 mM EDTA then resuspending them in divalent cation-free PBS supplemented with 0.5 mg/ml BSA. In the absence of divalent cations, SP1 cells did not adhere to FN. Addition of Ca^{2+}, and Mn^{2+} at low concentrations (0.1-1.0 mM) promoted adhesion of SP1 cells to FN, while concentrations greater than 1.0 mM were inhibitory and formed a precipitate. Mg^{2+} did not form a precipitate and was effective at promoting SP1 cell adhesion to FN over a wide range of concentrations (0.1-50 mM) (Figure 4). Other divalent cations such as Zn^{2+} or Cu^{2+} did not support SP1 cell adhesion to fibronectin (data not shown).

B) BMA5 antibody inhibits SP1 cell adhesion to fibronectin

SP1 cells express several integrin receptors with potential to bind fibronectin ($\alpha_5\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$). These integrins bind to their ligand via the RGD sequence. We therefore tested a GRGDSPK peptide and a control peptide GRGESPK to inhibit adhesion to FN. In our system, the GRGDSPK peptide was unable to reproducibly inhibit adhesion to fibronectin even at concentrations up to 1.0 mg/ml. However it was very efficient at inhibiting adhesion to vitronectin (data not shown). Blocking antibodies to many murine integrins are not available commercially, however, we obtained a blocking antibody to the murine $\alpha_5\beta_1$ integrin (gift from B. Chan). The BMA5 antibody was effective at inhibiting adhesion of SP1 cells to FN while the antibody in affinity purified form was also able to inhibit adhesion but was less efficient (Figure 5). The decrease in efficiency is likely a result of denaturation resulting from the purification process. A rabbit anti-mouse IgG control or the blocking antibody GoH3 (hybridoma conditioned medium) which recognizes the laminin binding integrin, $\alpha_6\beta_1$,

did not inhibit adhesion to FN. The GoH3 antibody was used as a control for the hybridoma conditioned medium (Figure 5).

C) role of extracellular matrix proteins in anchorage-independent growth of SP1 cells.

We have shown that SP1 cells adhere well to FN via the $\alpha_5\beta_1$ integrin and we have previously demonstrated that FN is abundant in SP1 colonies (Appendix I). Thus, the next step in investigating the role of extracellular matrix proteins, especially FN, in anchorage-independent growth was to remove the primary source of FN from the colonies. Fibronectin was partially removed from fetal bovine serum (FBS) by immunoprecipitation with anti-bovine FN antibodies (Telios). Partial removal of FN from serum reduced colony growth efficiency of SP1 cells by approximately 50% whereas endogenous IgG depletion with protein A Sepharose alone or preclearing of the serum with rabbit anti-mouse IgG did not remove any factors required for growth in soft agar (Figure 6). Addition of exogenous bovine plasma FN at 10 μg/ml to the FN reduced-serum partially reconstituted the colony growth. We have observed a reduction in colony growth in 4 of 6 experiments with a range of 26-89% inhibition. We have also analysed the immunoprecipitates of FN from serum on 6.0% SDS-PAGE under reduced conditions (data not shown). Although FN was likely not completely removed from the serum, our results show that we have removed sufficient FN from the serum to affect colony growth. Further experiments are required to determine the extent of the FN removed from the serum in our system by western blot analysis of the serum with FN removed by immunoprecipitation. It is possible that further depletion of FN will result in a greater and more consistant reduction in colony growth.

We next investigated whether addition of purified bovine plasma FN as well as other ECM proteins to limiting culture conditions (1% FBS) would enhance colony growth. When SP1 cells are cultured in agar containing 1% FBS, very few colonies grow and remain small in size as compared to those obtained in the presence of 7% FBS. In the complete absence of serum, SP1 cells do not form any colonies in soft agar. We used these limiting conditions (1% FBS) to determine which ECM proteins affect anchorage-independent growth of SP1 cells. The addition of FN and LM was shown to increase colony growth marginally in 1% FBS, but likely lacked the needed mitogenic components present in 7% FBS to generate large colonies (Figure 7). The increase

in colony growth observed with FN, although small, was reproducible in 4 of 4 experiments and the range of increase was from 29-63% increase compared to 1% FBS control. In contrast, both Coll I and IV inhibited colony growth of SP1 cells with the former being more effective. Collagen type I could inhibit colony growth in the presence of both 1% or 7% FBS (Figure 7, inset). Addition of Coll I or IV with FN or LM, respectively, also causes a reduction in colony growth demonstrating a dominant effect of Coll. We have observed a decrease in colony formation in the presence of Coll I in 4 of 7 experiments. In the 4 experiments where a decrease was observed, the range was from 46-96%.

We have previously shown that FN in SP1 colonies is present in the form of fibrils (Appendix I). We now show that the 70 amino-terminus kDa fragment of FN, which inhibits FN fibril formation by causing chain termination, reduces colony growth of SP1 cells. We have shown that addition of the 70 kDa fragment reduces colony growth by approximately 30% at a concentration of 10 μg/ml in agar as compared to a control 85 kDa peptide which does not inhibit FN fibril formation (Table 1). The 70 kDa fragment of FN does not inhibit SP1 cell adhesion to FN therefore its effect on colony growth is mediated through inhibition of fibril formation and not through interference of FN-integrin binding. We also incubated SP1 cells under anchorage-independent conditions with FITC-labelled FN for 5 days. These results show that exogenous FITC-labelled FN at 30 μg/ml was incorporated in SP1 colonies especially at the early stages, since FITC-labelled FN appears to be primarily in the central region of the colony (Figure 8).

2. Our second objective was to identify growth factors which stimulate anchorage-independent growth

A) TGF-β and HGF promote SP1 colony growth in 1% serum

We have shown that ECM proteins are required but not sufficient to maintain colony growth of SP1 cells. It is likely that growth factors are also required for adequate colony growth. SP1 cells respond to a number of growth factors including basic fibroblast growth factor (bFGF), PDGF, insulin-like growth factor (IGF), HGF, and transforming growth factor-beta (TGF-β). Although most of these factors have been shown to promote anchorage-independent growth of various cell lines, TGF-β is well established in the literature to promote anchorage-independent growth and modulate both ECM and integrin expression (Heino et al., 1989). In addition,

SP1 cells express high levels of the HGF receptor, c-Met, and secrete HGF therefore it is an important autocrine mitogenic factor for SP1 cells (Rahimi *et al.*, 1996b).

Thus, we investigated the ability of these two growth factors to promote anchorage-independent growth of SP1 cells. HGF at 5 ng/ml and TGF-β at 0.5 ng/ml both promoted anchorage-independent growth of SP1 cells in soft agar. However, TGF-β was considerably more efficient than HGF or FN in promoting colony growth in 1% FBS. When added in combination with FN, HGF or TGF-β showed a marginal additive effect compared to either growth factor alone (Figure 9). Concentrations of HGF greater than 10 μg/ml had a negative effect on SP1 colony growth (data not shown).

B) TGF-β increases adhesion of SP1 cells to the ECM

Transforming growth factor-β is well recognized in the literature for its ability to increase both extracellular matrix secretion and integrin expression (Heino *et al.*, 1989; Roberts *et al.*, 1992). We investigated whether TGF-β could promote adhesion of SP1 cells. SP1 cells were treated with TGF-β in the presence of 2% FBS under both adherent and anchorage-independent conditions. The serum concentration was reduced to 2% to minimize the effects of the serum but maintain viability of the cells in suspension cultures. When cells were treated with TGF-β under adherent conditions there was an increase in adhesiveness to FN, VN and Coll I and IV as determined by an adhesion assay (Figure 10A). SP1 cells did not adhere significantly to laminin with or without TGF-β treatment. When SP1 cells were treated with TGF-β under anchorage-independent conditions there was an increase in adhesiveness to Coll I and IV but the adhesiveness to FN, VN or LM remained unchanged (Figure 10B). In both cases the increase in adhesiveness to Coll I was greater than that to Coll IV. We are currently investigating the ability of TGF-β to alter ECM expression by Northern analysis and integrin expression by cell-surface labelling in SP1 cells under adherent and anchorage-independent conditions.

We also investigated whether HGF could alter the adhesive potential of SP1 cells. Our results showed that HGF does not alter the adhesive potential of SP1 cells on FN or VN but does stimulate cell spreading (data not shown). In addition, others have reported that HGF does not alter integrin expression (Matsumoto *et al.*, 1994).

3. Our third objective was to investigate how growth factors and integrins interact in anchorage-independent growth of SP1 cells

A) Effect of HGF, TGF-β and FN on anchorage-independent survival of SP1 cells.

We have shown above that FN, HGF, and TGF-β can increase colony formation of SP1 cells in agar. We next focused our cooperative interactions between integrins and growth factors on the effects of cell survival under anchorage-independent conditions.

We chose 1% FBS in our assay because it maintained a reasonable survival rate in the tested time period. Under serum free conditions, approximately 95% of the cells died after 24 h and we could only observe increases in cell survival. In the presence of 7% FBS, approximately 95% of the cells survived after 24 h and we could only observe decreases in cell survival. Using 1% FBS yielded a cell survival of 70-80% and permitted us to observe both increases in cell survival and death. We chose a 24 h time point to avoid effects resulting from the cells conditioning the medium. We have recently taken electron micrographs of SP1 cells and stained with bromodeoxyuridine, which detects fragmented DNA, in an attempt to determine whether the mechanism of cell death is apoptotic. Our initial results suggest that some SP1 cells undergo apoptosis under anchorage-independent conditions in low serum conditions (data not shown). Our results on demonstrated a small number of cells with classical apoptotic signs as compared to the cell death observed in survival assays. Further experiments are required to more definitively conclude the mechanism of cell death in SP1 cells maintained under anchorage-independent conditions.

We have shown that HGF at concentrations between 5-30 ng/ml can promote cell survival and that TGF-β at concentrations between 0.1-0.5 ng/ml is sufficient to promote survival of SP1 cells maintained in suspension. Concentrations of HGF higher than 30 ng/ml and TGF-β higher than 0.5 ng/ml resulted in a decrease in cell survival. Fibronectin could promote survival at concentrations greater than 1.0 μg/ml. Concentrations of FN greater than 10 μg/ml did not improve or reduce survival beyond the maximal effect of a 50% increase in survival (Figure 11A). In contrast to FN, Coll I and LM did not promote survival of SP1 cells under anchorage-independent conditions (Figure 11B). The 70 kDa fragment of FN which inhibits fibril formation but does not interfere with cell adhesion, could not promote survival and had no effect on survival mediated by FN (data not

shown). This result suggests that fibril formation is not required for survival under anchorage-independent conditions.

To determine whether the effect of FN on SP1 survival was mediated through the $\alpha_5\beta_1$ receptor, we tested the effect of BMA5 (anti-α_5 integrin blocking antibody) in our survival assay. The BMA5 antibody blocks adhesion by interfering with the $\alpha_5\beta_1$ integrin-ligand binding. However, a number of antibodies which bind to integrin subunits can cause conformational changes in the integrins which cause the generation of intracellular signals (activating antibodies), BMA5 is a newly developed antibody and inhibits $\alpha_5\beta_1$ mediated adhesion to FN (Fehlner-Grdiner et al., 1996), but no data is yet available on whether it possess activating properties. The BMA5 antibody (hybridoma conditioned medium) could significantly promote survival of SP1 cells in a 24 h assay as compared to the 1% FBS control. The increase in survival observed with BMA5 was similar to that observed with FN. Addition of the affinity purified BMA5 antibody at a concentration of 10 µg/ml could also significantly promote survival of SP1 cells under anchorage-independent conditions (Figure 12). In contrast, a rabbit anti mouse IgG control or a non-blocking anti-α_5 antibody (5H10) which recognizes the extracellular domain of the α_5 integrin subunit but does not interfere with ligand binding did not affect survival (Figure 12). Interestingly, addition of a secondary rabbit anti-mouse IgG which promote capping and internalization of the primary BMA5 antibody, resulted in an increase in cell death.

B) PI 3-kinase is required for FN + HGF-dependent cell survival

PI 3-kinase activity was shown to be required in HGF-mediated proliferation of adherent SP1 cells (Rahimi et al., 1996) and is involved in NGF mediated survival of adherent cells (Yao and Cooper, 1995 and 1996). We now investigated whether PI 3-kinase is involved in HGF and FN-mediated cell survival.

Individually, HGF and FN could promote cell survival by approximately 50% of the control (1% FBS), however when added in combination, HGF + FN resulted in an cooperative effect which was comparable to the survival observed with 7% FBS and significantly greater than FN or HGF alone (Figure 13). We are currently conducting survival experiments with BMA5 and HGF to test whether the cooperative effect of FN and HGF on survivalis mediated through the $\alpha_5\beta_1$.

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We used two approaches to determine whether PI 3-kinase is involved in survival of SP1 cells under anchorage-independent conditions. First, wortmannin, an inhibitor of PI 3-kinase, increased cell death in all groups including 7% FBS. However, the additive effect of both FN + HGF on cell survival was strongly inhibited by wortmannin, resulting in a survival similar to HGF or FN alone, which were only slightly reduced as compared to untreated controls (Figure 13). TGF-β could also reduce cell death by approximately 50% and acted in an additive fashion with FN (data not shown). Secondly, we used SP1 cells transfected with a dominant negative mutant of the p85 subunit of PI 3-kinase, designated Δp85 (obtained from M. Kasuga). The Δp85 protein lacks the binding site for the p110 catalytic subunit of PI 3-kinase. One of the clones isolated, designated C23, expresses high levels of Δp85, and shows reduced PI 3-kinase activity (Rahimi *et al.*, 1996). C23 cells showed no additive effect on survival when HGF and FN were added together. The increased cell survival in response to HGF or FN alone was only slightly affected. In contrast, another transfected clone, designated C22, which does not express high levels of Δp85 and maintains PI3-kinase activity, retained an additive effect of HGF + FN on SP1 cell survival (Figure 14). We have also performed preliminary experiments showing that the C23 clone is less efficient at forming colonies in agar (data not shown) Further experiments are required to ensure that this effect is not a result of clonal variation.

C) Phosphorylation of c-met, FAK and PI3-kinase

The biological effect (colony growth and survival) of FN and HGF on SP1 cells under anchorage-independent conditions implies that intracellular signals are transduced from these interactions. We investigated the tyrosine phosphorylation states of several candidate signaling molecules. We have previously demonstrated that HGF is a strong mitogen for SP1 cells under adherent conditions and that HGF and HGF receptor are co-expressed on SP1 cells (Rahimi *et al.*, 1996). We have also shown that HGF can increases colony growth of SP1 cells in agar. SP1 cells show continuous growth in serum-free medium consistent with an autocrine loop. We investigated the tyrosine phosphorylation of the HGF receptor under both adherent and anchorage-independent conditions. HGF receptor was constitutively phosphorylated on tyrosine residues in adherent SP1 cells. We observed only a slight increase in tyrosine phosphorylation upon addition of exogenous HGF at a concentration of

20 ng/ml. Tyrosine phosphorylation of HGF receptor was reduced under anchorage-independent conditions, but was restored in the presence of 20 ng/ml exogenous recombinant HGF (Figure 15A). The decrease in tyrosine phosphorylation was not a result of protein loading since both lanes contained equal amounts of HGF receptor (Figure 15B) When the blot was probed with anti-c-Met both the p145 c-Met protein and the p190 precursor were detected (Figure 15B), however only the p145 protein was tyrosine phosphorylated (Figure 15A).

Adhesion of many cell lines to ECM proteins results in the tyrosine phosphorylation of FAK, which is an important component in focal adhesion assembly and signal transduction pathways (Plopper *et al.*, 1995). We also observed that adhesion of SP1 cells to FN caused tyrosine phosphorylation of FAK which is increased in the presence of HGF, as reported by others (Matsumoto *et al.*, 1994). In contrast, soluble FN, HGF or FN + HGF together were unable to induce FAK phosphorylation above that observed in untreated SP1 cells maintained in suspension (Figure 16). We attempted to reprobe the blot with anti-FAK to check the FAK content in each lane, however our anti-FAK antibody was unable to detect the protein by western blotting analysis. However, the number of cells and the protein concentration were normalize in the lysate before immunoprecipitation.

PI 3-kinase is constitutively phosphorylated in SP1 cells adherent to poly-L-lysine and on FN, and showed little change in the level of tyrosine phosphorylation in response to HGF (Figure 17). Under anchorage-independent conditions PI 3-kinase showed a low level of tyrosine phosphorylation. We did not observe any consistent increase in PI 3-kinase phosphorylation in response to FN, HGF, or FN + HGF under anchorage-independent conditions. Preliminary results of SP1 cells grown under anchorage-independent conditions demonstrated that PI 3-kinase activity was increased in a stepwise manner in cell lysates of cells treated with FN, HGF, and FN + HGF compared to untreated cells (Figure 18).

Additional work pursued which was not included in original proposal.

4. Isolation and characterization of SP1 cell clones with differing anchorage-independent growth properties
A) Cl-12-H cells grow efficiently in agar and in serum-free medium.

We have cloned SP1 cells and tested 35 clones for their ability to grow in agar. We selected two clones

at opposing ends of the spectrum; Cl-12-H which grows very well in agar (approximately 40%) and forms large colonies with less variability in size and Cl-24-L which does not grow well in agar (approximately 2%) as compared to the parent cell line, SP1 (approximately 10%).

Our objective was to focus on differences in the adhesive properties of Cl-12-H and Cl-24-L and determine whether any observed differences correlated with their phenotypic differences in anchorage-independent growth. The morphology of Cl-12-H and Cl-24-L cells cultured on plastic in the presence of 7% FBS were very similar. The Cl-24-L cells show a more spread phenotype while Cl-12-H cells are more spindle shaped (Figure 19).

We compared the growth potential of the two clones with the parent population of SP1 cells. SP1 cells, Cl-12-H and Cl-24-L had similar growth rates when cultured in 7% FBS on tissue culture plastic as determined by ^{3}H-thymidine incorporation. However, in serum free conditions, the Cl-12-H clone incorporated significantly more ^{3}H-thymidine than either SP1 or the Cl-24-L cells (Figure 20).

Rahimi *et al.* (1996a) have shown that HGF is produced by SP1 cells and that they express the c-Met receptor. To determine whether the increased growth of Cl-12-H was a result of increased HGF-c-Met interaction, we used suramin (a non-specific inhibitor of growth factor receptor-ligand interactions). The growth, as measured by ^{3}H-thymidine incorporation, of all three cell lines was inhibited by suramin (500 μg/ml) in serum-free medium (data not shown) this result suggests that a secreted growth factor is responsible, however, suramin is none specific and a number of growth factors could be involved. We also examined the HGF receptor status on SP1 cells and the two clones by immunoprecipitation and western blot analysis. We did not observe any difference in HGF receptor expression and tyrosine phosphorylation on SP1, Cl-12-H and Cl-24-L cells (data not shown).

B) Cl-12-H cells adhere to collagen type I and express $\alpha_2\beta_1$

We investigated the adhesion profile of Cl-12-H and Cl-24-L cells. Cl-12-H and Cl-24-L cells adhere to a similar extent on FN and VN (data not shown). In contrast, Cl-12-H was able to adhere and spread on Coll I much more efficiently than Cl-24-L (Figure 21A and B). Cl-12-H cells were also able to adhere better to LM than SP1 or Cl-24-L cells (data not shown). Interestingly, both clones were able to adhere relatively well and to the same extent to Coll IV.

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We have shown that Cl-12-H cells adhere well to Coll I while Cl-24-L cells do not, therefore we investigated the expression of Coll receptors on the cell surface of the two clones. The primary integrin receptors for Coll are $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$. Immunoprecipitation of the integrin receptors for Coll using anti-α_2, α_3 and β_1 antibodies showed that Cl-12-H cells expressed higher levels of the $\alpha_2\beta_1$ integrin than Cl-24-L cells. Both cell lines expressed similar levels of the β_1 and the α_3 integrin (Figure 22A). In the α_2 immunoprecipitation we observed a band at approximately 110 kDa which co-migrates with the β_1 subunit but disappeared upon reduction of the samples (Figure 22B). We also observed a third band at approximately 100 kDa which is also present in the IgG control. This band was also lost after treatment with 2-mercaptoethanol. We do not have any antibodies which recognize the murine α_1 integrin subunit. However the α_1 subunit is 180 kDa in size compared to the other α subunits which are 120-130 kDa in size. Based on this difference, the α_1 subunit can be distinguished from the other α subunits in the β_1 immunoprecipite. We did not observe any α_1 integrin subunit in the β_1 immunoprecipite. We have not determined whether $\alpha_2\beta_1$ is the primary receptor for collagen or what is the contribution of other Coll receptors such as $\alpha_3\beta_1$ and $\alpha_v\beta_3$ since we do not have any specific blocking antibodies or peptides to these mouse integrin receptors.

C) Fibronectin and collagen type I promote anchorage-independent growth of Cl-12-H cells

We have previously shown that FN can marginally increase colony growth of SP1 cells in soft agar in the presence of 1% FBS, while Coll I inhibits this growth. We tested the effect of FN, LM, VN and Coll I on colony growth in agar of Cl-12-H and Cl-24-L cells. Similar to the results observed with SP1 cells, FN and LM were found to promote colony growth of Cl-12-H cells. When added in combination, FN + LM or FN + Coll I showed an additive increase in colony growth (Figure 23A). Interestingly, collagen type I which inhibits colony growth of SP1 cells promotes that of Cl-12-H cells and could also increase colony growth in the presence of 7% FBS in a concentration dependent manner (Figure 23B). Neither FN or Coll I could affect growth of Cl-24-L cells in agar (Figure B). In an attempt to evaluate the role of integrin expression on anchorage-independent growth, we, in collaboration with Dr. Bosco Chan, transfected the α_2 subunit in SP1 cells. However, we have had difficulty isolating a positive clone. It appears that the transfected cells do not remain viable in culture. Control experiments

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are underway to try and determine the reason for unviable transfectants

In conclusion, we have isolated an SP1 clone which is very efficient at anchorage-independent growth, as well as growth in serum-free conditions. Cl-12-H cells also adhered to Coll I and expresses $\alpha_2\beta_1$ integrin. A summary of the properties of Cl-12-H, Cl-24-L and SP1 cells are presented in Table 2.

Conclusions

Key novel findings obtaining from this project: There are two novel observations obtained in this work;

1. A number of reports have shown the presence of ECM proteins in tumor spheroids. However this is the first report where individual ECM proteins in spheroids were investigated directly. We show by partially removing FN from tissue culture medium with optimal growing conditions (7% FBS), and by adding FN to tissue culture medium with limiting growth conditions (1% FBS), that FN is required for anchorage-independent survival and growth of SP1 cells. In contrast, Coll I inhibited colony growth of SP1 cells which express very low levels of the Coll receptor $\alpha_2\beta_1$. The SP1 cell clone, Cl-12-H, which expresses higher levels of $\alpha_2\beta_1$ showed increased colony growth in response to Coll I. Thus, different ECM proteins are capable of promoting growth, providing the appropriate cell surface receptors are present and in sufficient numbers. Our results also show that ECM-integrin interactions alone can not induce anchorage-independent growth of SP1 cells. They can only modulate anchorage-independent growth in cells with the potential to grow under these conditions.

2. Previous work performed on the role of ECM proteins and growth factors in cell survival has been performed in adherent normal or transformed cells. This is the first report investigating the role of ECM protein and growth factors in tumor cell growth and survival under anchorage-independent conditions. We have demonstrated a cooperative role for FN and HGF in cell survival under anchorage-independent conditions. In addition, we have shown that PI 3-kinase is required in FN + HGF mediated survival under anchorage-independent conditions as demonstrated by using wortmannin, a PI 3-kinase inhibitor and cells transfected with a dominant negative p85 subunit of PI 3-kinase.

Discussion

ECM in colony growth

Adhesion to ECM appears (at least superficially) to be the only parameter different between adherent and anchorage-independent cells. It is conceivable that some tumor cells may be able to grow in the complete absence of ECM, however, the cells' ability to respond to ECM proteins and its particular response to ECM may be a key regulator of anchorage-independent growth. Our work, as well as that of others, has shown the presence of ECM proteins in tumor spheroids. Here we provide evidence that ECM proteins are required for growth under anchorage-independent conditions. We have shown that FN is required for SP1 colony growth. Although FN is not sufficient to stimulate colony formation, it appears to play an important role in regulating growth. In support of these results, Coucke *et al* (1992) have shown that preincubation of B16 melanoma cells with FN can increase the formation of lung metastases when injected into mice. In addition, expression of FN is often increased in the stroma of mammary carcinomas (Christensen, 1992). Thus ECM-integrin interactions do play a role in colony formation in our system and may play a role *in vivo*.

We have also investigated the role of FN fibrils in SP1 colony growth. Inhibition of FN fibril formation with a 70 kDa fragment had no effect on FN-dependent survival but did reduce colony growth. It appears that FN (or collagen) fibril assembly may provide a physical structure for cells, which permit changes in shape and result in different responses to growth factors. In support of this statement, Re *et al* (1994) observed that integrin ligation was not sufficient to inhibit apoptosis of human endothelial cells. Soluble VN could not inhibit apoptosis of these cells, they required to undergo a minimal degree of shape change in order to survive. Thus, tumor cells may have escaped the requirements for cell shape to survive but still require some shape changes to proliferate. These changes are provided by the matrix fibrils or interactions with other cells in the tumor spheroid. In addition, we have shown that Coll I can support anchorage-independent growth of Cl-12-H cells. However, we have yet to investigate whether collagen type I is assembled in fibrils in SP1 colonies. Collagen type I fibril assembly is an entropic process, while FN fibril assembly requires cell surface receptors. Thus the mechanisms by which FN or Coll I fibrils support survival and growth may differ.

An exogenous source of ECM proteins may be most critical at the early stages of colony formation. We

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have shown that FITC-labelled FN appears to be predominantly located in the central region of the colony. Once the colony has started and matrix fibrils are established, the cells may change their environment by secreting their own ECM proteins and growth factors such as TGF-β which may, in turn, affect ECM production of surrounding cells. The cells would then be less dependent on exogenous factors. Further experiments are required to determine the kinetics for matrix requirements. Removal of exogenous ECM protein at various time points and following the kinetics of colony growth would establish whether exogenous ECM proteins are required in the early stages of colony formation.

In conclusion, tumor cells can respond to ECM proteins under anchorage-independent conditions; they simply do not spread. SP1 cells can synthesize their own ECM and growth factors under adherent conditions but still require exogenous factors under anchorage-independent conditions, suggesting a shift from autocrine to paracrine mechanisms under anchorage-independent conditions. Once the colony is established, and FN fibril interactions occur, there may be another shift back from paracrine to autocrine growth factor requirements. These data suggest that tumor cells are vulnerable as single cells since they have a high dependence on exogenous factors for survival. In support of this statement, few cells which disseminate from the primary tumor actually go on to form secondary metastases (Weiss *et al.*, 1982). Figure displays a scenario of how ECM and growth factors are involved when an adherent cell becomes anchorage-independent and forms a tumor spheroid.

Integrins in anchorage-independent growth

Howlett *et al.* (1995) have suggested that in mammary carcinomas, the interactions between ECM and integrins are disturbed. The right integrins are expressed on the cell surface but the correct differentiation signals are not generated from them. Howlett *et al.* (1995) have shown that Matrigel promoted acinar structure formation by normal mammary cells could be inhibited with anti-ß$_1$ integrin antibodies, while mammary carcinoma cells formed disorganized spheroids in Matrigel which could not be inhibited by anti-ß$_1$ antibodies. They suggested that growth of mammary tumor spheroids was integrin-independent. In contrast, Matrigel increased the metastatic ability of mammary carcinoma cell lines when co-injected with carcinoma cells subcutaneously into nude mice (Mullen *et al.*, 1996). These two results appear to be in conflict with each other. One suggests that tumor cell colony growth is independent of the ECM-integrin interactions while the other suggests that ECM promotes

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metastasis hence the metastatic process is ECM-dependent. Close analysis of the results by Howlett *et al.* (1995) shows that addition of the anti-β_1 antibodies caused a slight increase in growth of the tumor cells instead of inhibition as seen in normal cells. It is possible that the anti-β_1 antibody is inhibitory in normal cells because they do not recognize the antibody as their natural ligand. In contrast the integrins in transformed cells may not need the specificity provided by the natural ligand and are stimulated by the anti-β_1 antibody as a result of changes in the activation state of the integrin. These changes in activation state are likely caused by changes in the activity of cytoplasmic signaling proteins, membrane proteins which alter integrin function or changes in phospholipid composition of the plasma membrane. This is further supported by Werb *et al.* (1989) who demonstrated that synovial fibroblasts adherent to anti-β_1 antibodies or fragments of FN secreted the proteases stromelysin and collagenase while they did not on intact FN. In some cases an intact ECM molecule may ligate several integrin molecules simultaneously while ECM fragments ligate only one, thereby generating a different signal. These results suggest that parts of the ECM protein are activating while other parts may be suppressive. It was later found that signaling through the $\alpha_5\beta_1$ receptor which recognizes the 120 kDa fragment of FN, increased metalloproteinase expression, while signaling through the $\alpha_4\beta_1$ receptor, which recognizes a different part of FN (CS-1 region) suppressed expression (Huhtala *et al.*, 1995). In addition, proteolytic cleavage of Coll I exposes a cryptic RGD sequence to the $\alpha_v\beta_3$ integrin which is not available on native Coll (Montgomery *et al.*, 1994). It is also known that the ability of integrins to recognize their ligands is regulated by delicate alterations in the conformation of the molecule regulated by divalent cations and intracellular proteins (reviewed in Mould, 1996). In addition, the response of an integrin to an ECM protein may be a result of other factors such as ECM proteins or growth factors which are present in the environment. Together, these results support Howlett *et al* (1995) theory that integrins on transformed cells do not properly recognize their ligands as they showed with human mammary cells.

Although our interest was in the $\alpha_5\beta_1$- fibronectin system on SP1 colony growth, we obtained several results which suggested a role of the $\alpha_2\beta_1$- collagen receptor system in colony growth. (1) The $\alpha_2\beta_1$ integrin expression was increased on the cell surface under anchorage-independent conditions. (2) SP1 cells under anchorage-independent conditions treated with TGF-β only increase their adhesion to collagen (3) SP1 cells which are selected for their anchorage-independent growth properties expressed increased $\alpha_2\beta_1$ expression and collagen could promote

their growth in agar.

Tumor cells typically maintain the normal integrin profile but show a reduced or disorganized expression of integrins (Alford and Taylor-Papadimitriou, 1996; Berdichevsky $et\ al.$, 1994). *De novo* expression or complete loss of integrin expression are seen occasionally in transformed cells. Integrin expression is important for mammary cell function. For example, the $\alpha_2\beta_1$ integrin is required for mammary cell morphogenesis in a collagen gel, while the $\alpha_3\beta_1$ integrin is required for morphogenesis in a laminin rich matrix (Howlett $et\ al.$, 1995). These data show that different integrins are capable of mediating mammary cell morphogenesis depending on the ECM protein present in the extracellular environment. Saelman $et\ al$ (1995) have demonstrated that loss of $\alpha_2\beta_1$ integrin reduces the ability of MDCK cells to survive and form cysts in three-dimensional collagen gels. The cells lacking $\alpha_2\beta_1$ also demonstrated reduced tubulogenesis and branching morphogenesis in response to HGF. This result also shows a cooperative effect between integrins and growth factors under anchorage-independent conditions. These results (Howlett $et\ al.$ 1995) suggest that it is not a particular integrin receptor which is responsible for increased anchorage-independent growth, but likely several receptors are capable of supporting growth providing that the appropriate ligand is present. Our results support the idea that multiple integrins are involved, since both $\alpha_5\beta_1$ or $\alpha_2\beta_1$ could support anchorage-independent growth in Cl-12-H cells. Cl-12-H cells which express higher levels of $\alpha_2\beta_1$ can respond to Coll I while SP1 and Cl-24-L cells which express lower levels of this receptor do not increase colony growth in response to Coll. Again it appears that the level of receptor expression is an important parameter in determining its function. In support of this observation, overexpression of $\alpha_2\beta_1$ in mammary cells tends to restore the normal phenotype. However it is difficult to assess exactly how many receptors are required especially considering that other factors in the environment and proteins on the cell surface may contribute to the response.

Growth factors and anchorage-independent growth

TGF-β can increase ECM and integrin expression in a number of cell lines and can negatively regulate growth by stimulating cyclin dependent kinase inhibitors (Polyak $et\ al.$, 1994; Reynisdóttir $et\ al.$, 1995). TGF-β often has an opposing effect on growth in transformed cells (Welch $et\ al.$, 1990). It is possible that TGF-β regulates anchorage-independent growth by one of three mechanisms (1) by altering the ECM and integrin expression (2)

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direct signaling through the receptor affecting cyclins and cdk's or (3) a combination of both. TGF-β can inhibit cell growth of adherent SP1 cells but promotes anchorage-independent growth of these cells in agar. More than just signaling from integrins and TGF-β receptors is required for TGF-β-mediated anchorage-independent growth since FN cannot replace the adhesive signal in suspension. A combination of FN and TGF-β show an additive effect on SP1 colony growth. Cell shape and other parameters mediated by cell adhesion may also contribute to the effects of TGF-β on cell growth (Sutton *et al.*, 1991). Roskelley *et al* (1994) have shown that ECM proteins (Matrigel) are required for milk production in mammary epithelial cells maintained in a rounded morphology on polyHEMA. Adherent cells were much less efficient at expressing β-casein. The effects of individual matrix components or the use of transformed cells were not determined in this model. It is also unclear to what extent a single matrix protein can contribute compared to several components as seen in experiments with Matrigel.

HGF is a pleiotropic factor reported to affect many cellular functions. It is reported not to affect ECM or integrin expression, however the cellular response to HGF is different depending of the ECM present (Taipale and Keski-Oja, 1996). HGF is also an important regulator of mammary cell growth and morphogenesis. Taking into consideration the last two points, a change in ECM may lead to a change in HGF response and cause a change from HGF-mediated mammary cell morphogenesis to HGF-mediated cell growth. Mammary tumors often express elevated levels of FN and Coll I in the stroma, hence these changes may be accompanied by changes in HGF-mediated signaling. It remains to be determined exactly what parameters are required to regulate HGF-mediated morphogenesis versus growth in mammary cell culture models.

Normal cells in suspension fail to undergo DNA synthesis in the presence of growth factors but show increased expression of c-Myc (Barrett *et al.*, 1995). It was shown that cyclin A production is required for progression through the cell cycle and that fibroblasts in suspension fail to increase cyclin A expression, therefore cyclin A is adhesion dependent. In contrast, Cyclin D and E are more growth factor dependent. Interestingly, TGF-β can override the adhesion requirements in some fibroblasts (Han *et al.*, 1993). One can see a cooperative effect between growth factors and cell adhesion at the level of the cyclins. We have also seen that c-Src, FAK, Ras and PI 3-kinase can function in both integrin and growth factor signaling pathways, however there still remain many unanswered questions regarding the mechanisms by which growth factor and integrin signaling pathways interact.

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Signaling in anchorage-independent growth

One issue which remains to be determined is the specificity of protein-protein interactions after integrin-dependent activation of signal transduction pathways. For example FAK can bind to more than 9 substrates including PI 3-kinase, Src, Csk, HGF receptor, talin, paxillin, p130^{cas}, Grb2 and β_1 integrin. It is likely that FAK does not bind to all these substrates every time it is activated. It may also have different affinities for each substrate. Therefore which substrate is activated will depend on the abundance of FAK, its ligand and its affinity to the particular substrate. When this protein is over-expressed by transfection or naturally through transcriptional regulation, there is a sufficient amount of FAK for both high and low affinity substrates to interact with FAK. This may activate signaling pathways which may not usually be activated under these conditions. Such a mechanism is more clearly seen with integrin expression, where upregulation and downregulation of integrins at the cell surface change the function of the cell. The observation that transfection of the α_5 integrin in CHO cells reduces the transformed phenotype suggests two possible mechanisms: (1) The number of receptors on the cell surface may play an important role in which signals are generated and (2) the signals generated from the integrins are dependent on other signals, hence opposing signals can be generated from the same receptor depending on which other signaling pathways or molecules are active. In support of this evidence, Varner *et al* (1995) demonstrated that unligated receptor generated negative signals for growth while ligated receptor generated positive signals for growth. There is also increasing evidence that there are cooperative interactions between different integrin receptors and between growth factors and integrin signaling pathways. In contrast we show that high expression of $\alpha_5\beta_1$ promotes colony growth. However it may be that other parameters are also involved or the level of expression of $\alpha_5\beta_1$ in our cells is still not as high as compared to transfected cells. The absolute number of receptors per cell would have to be calculated. It would be interesting to compare various integrin signaling mechanisms based on the level of integrin expression on the cell surface. This could be achieved by using the tetracycline inducible promoter in which expression can be regulated by the concentration of tetracycline added.

In conclusion

Transformation is a stepwise process which occurs after many mutations. Cells are therefore becoming

gradually transformed. At early stages, ECM-integrin interactions may promote differentiation while at later stages, promote migration and invasion during progression to the transformed and metastatic phenotype. Normal and transformed cells respond to the ECM under non-adherent conditions but it is the interpretation of the cellular signals generated as a result of ECM-integrin interactions which determines whether the cell will differentiate or proliferate. These signals depend on a number of factors (1) degree of differentiation of the cells, (2) the quality and quantity of cell surface receptors, (3) the presence and type of growth factors, (4) cell type and (5) cell shape. With a full understanding of all the parameters it would seem possible that transformed cells with different degrees of malignancy can be stimulated to redifferentiate providing the correct environment is provided.

The big picture

One has to be cautious in extrapolating results obtained *in vitro* to an *in vivo* situation since there are many other factors present *in vivo* which affect tumor cell function and phenotype. Here I describe an *in vivo* metastatic cascade and describe at what stages the findings of this work may contribute.

Tumor cells grow at the site of origin until the tumor becomes vascularized, then the tumor cells escape into the circulation. Once in the circulation, the cells must survive under anchorage-independent conditions and extravasate at a distant site and grow into a secondary tumor or metastasis. The properties of metastatic cells are therefore different from the primary tumor cells.

This work makes several contributions on the understanding of this process. Once cells escape from the primary tumor, and enter the circulation, factors in the serum such as ECM proteins or growth factors act as survival factors for some tumor cells (i.e. few cells can respond to these factors). We have shown that FN, HGF and TGF-β support survival of SP1 cells, however, other serum factors are likely also support this function *in vivo* depending on the specific phenotype of the tumor cell. The increase in the $\alpha_2\beta_1$ integrin (laminin and collagen receptor) would be useful in adhesion to the basement membrane at a distant site which is primary composed of collagen type IV and laminin. Once it has extravasated at a distant site, the tumor may grow slowly at first, but will become vascularized. Since new blood vessels tend to be very leaky, they would provide plenty of plasma proteins such as FN for tumor growth. For tumor cells which do not express sufficient fibronectin receptors, expression of collagen receptors could substitute and exogenous source of collagen could be provided by the

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newly formed endothelial cells or surrounding stromal fibroblasts. Our results also demonstrate that survival and anchorage-independent growth are separate steps and that fibril formation is not required for cell survival but is required for colony growth.

This work has made some contributions to the understanding of the role of ECM protein in anchorage-independent growth of a murine mammary carcinoma cell line. An understanding of the growth requirements of tumor cells in spheroids will improve our ability to treat metastatic disease, the primary cause of mortality in breast cancer.

References

Adams,J.C. and Watt,F.M. Changes in keratinocyte adhesion during terminal differentiation: reduction in fibronectin binding precedes $\alpha_5\beta_1$ integrin loss from the cell surface. Cell. 63:425-435, 1990.

Akiyama,S.K. and Yamada,K.M. The interaction of plasma fibronectin with fibroblasts in cell suspension. J. Biol. Chem. 260:4492-4500, 1985.

Albelda,S.M. and Buck,C.A. Integrins and other cell adhesion molecules. FASEB J. 4:2868-2880, 1990.

Alford,D. and Taylor-Papadimitriou,J. Cell adhesion molecules in the normal and cancerous mammary gland. J. Mammary Gland Biol. Neo. 1:207-218, 1996.

Barrett,J.F., Lewis,B.C., Hoang,A.T., Alvarez,R.J. Jr, and Dang,C.V. Cyclin A links c-myc to adhesion-independent cell proliferation. J. Biol. Chem. 270:15923-15925, 1995.

Berdichevsky,F., Wetzels,R., Shearer,M., Martignone,S., Ramaekers,F.C.S. and Taylor-Papadimitriou,J. Integrin expression in relation to cell phenotype and malignant change in the human breast. Mol. Cell. Differ. 2:255-274, 1994.

Boudreau,N., Sympson,C.J., Werb,Z. and Bissell,M.J. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science. 267:891-893, 1995.

Boudreau,N., Werb,Z., and Bissell,M.J. Suppression of apoptosis by basement membrane requires three-dimensional tissue organization and withdrawal from the cell cycle. Proc. Natl. Acad. Sci. USA. 93:3509-3513, 1996.

Chen,D., Magnuson,V., Hill,S., Arnaud,C., Steffensen,B. and Klebe,R.J. Regulation of integrin gene expression by substrate adherence. J Biol. Chem. 267:23502-23506, 1992.

Christensen,L. The distribution of fibronectin, laminin and tetranectin in human breast cancer with special attention to the extracellular matrix. APMIS. Suppl. 26:6-39, 1992.

Coucke,P., De Pauw-Gillet,M.C., De Leval.L., Noel,A., Bassleer,R. and Foidart,J.M. Fibronectin promotes lung colony formation in the mouse by B16 melanoma cells spheroids. In vivo. 6:481-486, 1992.

Dalton,S.L., Marcantonio,E.E. and Assoian,R.K. Cell attachment controls fibronectin and $\alpha_5\beta_1$ integrin levels in fibroblasts. Implications for anchorage-dependent and -independent growth. J. Biol. Chem. 267:8186-8191, 1992.

Dedhar,S. Integrins and tumor invasion. Bioassays. 12:1-8, 1990.

Dedhar,S. and Saulnier,R. Alterations in integrin receptor expression on chemically transformed human cells. Specific enhancement of laminin and collagen receptor complexes. J. Cell Biol. 110:481-489, 1990.

Elliott,B.E., Maxwell,L., Arnold,M., Wei,W.Z. and Miller,F.R. Expression of epithelial-like markers and class I major histocompatibility antigens by a murine carcinoma growing in the mammary gland and in metastases: Orthotropic site effects. Cancer Res. 48:7237-7245, 1988.

Fehlner-Gardiner,C.C., Uniyal,S., von Ballestrem,C.G. and Chan,B.M.C. Differential utilization of VLA-4 ($\alpha4\beta1$) and -5 ($\alpha5\beta1$) integrins during the development of mouse bone marrow-derived mast cells. Differentiation. 60:317-325, 1996.

Frisch,S.M. and Francis,H. Disruption of epithelial cell-matrix interactions induces apoptosis. J. Cell Biol. 124:619-626, 1994.

Frisch,S.M., Vuori,K., Ruoslahti,E. and Chan-Hui,P.Y. Control of adhesion-dependent cell survival by focal adhesion kinase. J. Cell Biol. 134:793-799, 1996.

Giancotti,F.G. and Mainiero,F. Integrin-mediated adhesion and signaling in tumorigenesis. Biochim. Biophys. Acta. 1198:47-64, 1994.

Glimelius,B., Norling,B., Nederman,T. and Carlsson,J. Extracellular matrices in multicellular spheroids of human glioma origin: increased incorporation of proteoglycans and fibronectin as compared to monolayer cultures. APMIS. 96:433-444, 1988.

Han,E.K.H., Guadagno,T.M., Dalton,S.L. and Assoian,R.K. A cell cycle and mutational analysis of anchorage-independent growth: cell adhesion and TGF-β_1 control G1/S transit specifically. J. Cell Biol. 122:461-471, 1993.

Hauptmann,S., Denkert,C., Löhrke,H., Tietze,L., Ott,S., Klosterhalfen,B. and Mittermayer,C. Integrin expression on colorectal tumor cells growing as monolayers, as multicellular tumor spheroids, or in nude mice. Int. J. Cancer. 61:819-825, 1995.

Heino,J., Ignotz,R.A., Hemler,M.A., Crouse,C. and Massagué,J. Regulation of cell adhesion receptors by transforming growth factor-β. Concominant regulation of integrins that share a common β_1 subunit. J. Biol. Chem. 265:380-388, 1989.

Howlett,A.R., Bailey,N., Damsky,C.H., Petersen,D.W. and Bissell,M.J. Cellular growth and survival are mediated by β_1 integrins in normal human breast epithelium but not in breast carcinoma. J. Cell Sci. 108:1945-1957, 1995.

Huhtala,P., Humphries,M.J., McCarthy,J.B., Tremble,P.M., Werb,Z. and Damsky,C.H. Cooperative signaling by $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. J. Cell Biol. 129:867-879, 1995.

Hynes,R.O. Integrins: A family of cell surface receptors. Cell. 48: 549-554, 1987.

Isberg,R.R. and Leong,J.M. Multiple β_1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell. 60: 861-871, 1990.

Johansson,S. Demonstration of high affinity fibronectin receptors on rat hepatocytes in suspension. J. Biol. Chem. 260:1557-1561, 1985.

Kang,J.S. and Krauss,R.S. Ras induces anchorage-independent growth by subverting multiple adhesion-regulated cell cycle events. Mol. Cell. Biol. 16:3370-3380, 1996.

Kim,K., Akoto-Amanfu,E., Cherrick,H.M. and Park,N.H. Anchorage-independent growth and the expression of cellular proto-oncogenes in normal human epidermal keratinocytes and in human squamous cell carcinoma cell lines. Oral Surg. Oral Med. Oral Path. 71:303-311, 1991.

Masuda,A., Kizaka-Kondoh,S., Miwatani,H., Terada,Y., Nojima,H. and Okayama,H. Signal transduction cascade shared by epidermal growth factor and platelet-derived growth factor is a major pathway for oncogenic transformation in NRK cells. New Biol. 4:489-503, 1992.

Matsumoto,K., Ziober,B.L., Yao,C.C. and Kramer,R.H. Growth factor regulation of integrin-mediated cell motility. Cancer Metastasis Rev. 14:205-217, 1995b.

McNamee,H.P., Ingber,D.E. and Schwartz,M.A. Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. J. Cell Biol. 121:673-678, 1993.

Meredith,J.E., Fazeli,B. and Schwartz,M.A. The extracellular matrix as a cell survival factor. Mol. Biol. Cell.

4:953-961, 1993.

Montgomery,A.M.P., Reisfeld,R.A. and Cheresh,D.A. Integrin $\alpha_v\beta_3$ rescues melanoma cells from apoptosis in three-dimensional dermal collagen. Proc. Natl. Acad. Sci. USA. 91:8856-8860, 1994.

Mould,P.A. Getting integrins into shape: recent insights into how integrin activity is regulated by conformational changes. J. Cell Sci. 109:2613-2618, 1996.

Mullen,P., Ritchie,A., Langdon,S.P. and Miller,W.R. Effect of matrigel on the tumorigenicity of human breast and ovarian carcinoma cell lines. Int. J. Cancer. 67:816-820, 1996.

Nederman,T., Norling,B., Glimelius,B., Carlsson,J. and Brunk,U. Demonstration of an extracellular matrix in multicellular tumor spheroids. Cancer Res. 44: 3090-3097, 1984.

Olive,P.L. and Durand,R.E. Drug and radiation resistance in spheroids: cell contact and kinetics. Cancer Metastasis Rev. 13:121-138, 1994.

De Pauw-Gillet,M.C.A.J., Christiane,Y.M., Foidart,J.M. and Bassleer,R.J.B. Analysis of tridimensional mixed cultures of mouse B16 melanoma cells and 3T3 fibroblasts. Anticancer Res. 8:153-160, 1988.

Plopper,G.E., McNamee,H.P., Dike,L.E., Bojanowski,K. and Ingber,D.E. Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. Mol. Biol. Cell. 6:1349-1365, 1995.

Polyak,K., Kato.J., Solomon,M.J., Sherr,C.J., Massagué,J., Roberts,J.M. and Koff,A. p27^{Kip1}, a cyclin-Cdk inhibitor, links transforming growth factor-β
and contact inhibition to cell cycle arrest. Genes Devel. 8:9-22, 1994.

Pullan,S., Wilson,J., Metcalfe,A., Edwards,G.M., Goberdhan,N., Tilly,J., Hickman,J.A., Dive,C. and Streuli,C.H. Requirement of basement membranes for the suppression of programmed cell death in mammary epithelium. J. Cell Sci. 109:631-642, 1996.

Rahimi,N., Tremblay,E., McAdam,L., Park,M., Schwall,R. and Elliott,B.E. Identification of hepatocyte growth factor autocrine loop in a murine mammary carcinoma. Cell Growth and Diff. 7:263-270, 1996a.

Rahimi,N., Tremblay,E. and Elliott,B.E. Phosphatidylinositol 3-kinase activity is required for hepatocyte growth factor-induced mitogenic signals in epithelial cells. J. Biol. Chem. 271:24850-24855, 1996b.

Rak,J., Mitsuhashi,Y., Erdos,V., Huang,S., Filmus,J. and Kerbel,R.S. Massive programmed cell death in intestinal epithelial cells induced by three-dimensional growth conditions: suppression by mutant c-H-ras oncogene expression. J. Cell Biol. 131:1587-1598, 1995.

Re,F., Zanetti,A., Sironi,M., Polentarutti,N., Lanfrancone,L., Dejana,E. and Colotta,F. Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. J. Cell Biol. 127:537-546, 1994.

Reynisdóttir,I., Polyak,K., Iavarone,A. and Massagué,J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-β. Genes Devel. 9:1831-1845, 1995.

Rizzino,A., Ruff,E. and Rizzino,H. Induction and modulation of anchorage-independent growth by platelet-derived growth factor, fibroblast growth factor and transforming growth factor-β1. Cancer Res. 46:2816-2820, 1986.

Roberts,A.B., McCune,B.K. and Sporn,M.B. TGF-β:regulation of extracellular matrix. Kidney Inter. 41:557-559, 1992.

Rodgers,H.F., Lavranos,T.C., Vella,C.A. and Rodgers,R.J. Basal lamina and other extracellular matrix produced by bovine granulosa cells in anchorage-independent culture. Cell Tissue Res. 282:463-471, 1995.

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Roskelley,C.D. and Bissell,M.J. Dynamic reciprocity revisited: a continuous, bidirectional flow of information between cells and the extracellular matrix regulates mammary epithelial cell function. Biochem. Cell Biol. 73:391-397, 1995.

Roskelley,C.D., Desprez,P.Y. and Bissell,M.J. Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. Proc. Natl. Acad. Sci. USA. 91:12378-12382, 1994.

Ruoslahti,E. Integrins. J. Clin. Invest. 87:1-5, 1991.

Ruoslahti,E. and Reed,J.C. Anchorage dependence, integrins,and apoptosis. Cell. 77:477-478, 1994.

Saelman,E.U.M., Keely,P.J. and Santoro,S.A. Loss of MDCK cell $\alpha_2\beta_1$ integrin expression results in reduced cyst formation, failure of hepatocyte growth factor/scatter factor-induced branching morphogenesis, and increased apoptosis. J. Cell Sci. 108:3531-3540, 1995.

Schubert,D. Collaborative interactions between growth factors and the extracellular matrix. TIBS. 2:63-66, 1992.

Shin,S.I., Freedman,V.H., Risser,R. and Pollack,R. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage-independent growth *in vitro*. Proc. Natl. Acad. Sci. USA. 72:4435-4439, 1975.

Sutton,A.B., Canfield,A.E., Schor,S.L., Grant,M.E. and Schor,A.M. The response of endothelial cells to TGF-$\beta 1$ is dependent upon cell shape, proliferative state and the nature of the substratum. J. Cell Sci. 99:777-787, 1991.

Taipale,J. and Keski-Oja,J. Hepatocyte growth factor releases epithelial and endothelial cells from growth arrest induced by transforming growth factor-$\beta 1$. J. Biol. Chem. 271:4342-4348, 1996.

Tavoloni,N., Inoue,H., Sabe,H. and Hanafusa,H. v-src transformation of rat embryo fibroblasts. Inefficient conversion to anchorage-independent growth involves heterogeneity of primary cultures. J. Cell Biol. 126:475-483, 1994.

Varner,J.A., Emerson,D.A., and Juliano,R.L. Integrin $\alpha_5\beta_1$ expression negatively regulates cell growth: reversal by attachment to fibronectin. Mol. Biol. Cell. 6:725-740, 1995.

Waleh,N.S. Gallo,J. Grant,T.D., Murphy,B.J., Kramer,R.H. and Sutherland,R.M. Selective down-regulation of integrin receptors in spheroids of squamous cell carcinoma. Cancer Res. 54:838-843, 1994.

Welch,D.R., Fabra,A. and Nakajima,M. Transforming growth factor-β stimulates mammary adenocarcinoma cell invasion and metastatic potential. Proc. Natl. Acad. Sci. USA. 87:7678-7682, 1990.

Werb,Z., Tremble,P.M., Behrendtsen,O., Crowley,E. and Damsky,C.H. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. 109:877-889, 1989.

Weiss,L., Mayhew,E., Glaves Rapp,D. and Holmes,J.C. Metastatic inefficiency in mice bearing B16 melanomas. Br. J. Cancer. Suppl. 45-53, 1982.

Yao,R. and Cooper,G.M. Requirement for phosphatidylinositol 3-kinase in the prevention of apoptosis by nerve growth factor. Science 267:2003-2006, 1995.

Yao,R. and Cooper,G.M. Growth factor-dependent survival of rodent fibroblasts requires phosphatidylinositol 3-kinase but is independent of p70[S6K] activity. Oncogene. 13:343-351, 1996.

Zhang,K., Vuori,K., Reed,J.C. and Ruoslahti,E. The $\alpha_5\beta_1$ integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. Proc. Natl. Acad. Sci. USA. 92:6161-6165,1995.

Table 1

Effect of the 70 kDa and 85 kDa fragments of fibronectin on SP1 cell colony growth

Fibronectin fragments	Number of colonies
3% FBS	90 ± 6.5
3% FBS + 70 kDa FN Fragment	62 ± 4.1
3% FBS + 85 kDa FN Fragment	91 ± 11

Legend:
SP1 were seeded (30,000/well) in 0.75 ml of a 1:1 mixture of agar and RPMI 1640 medium in 35 x 10 mm dishes. The final concentration of agar was 0.36%. The 70 kDa N-terminal fragment or the 85 kDa fragment were added to the mixture at 10 μg/ml before solidification. The dishes were incubated at 37°C for 10 days. The colonies were then fixed in methanol, stained with Giemsa and counted manually. The results are expressed as the mean $\pm$ SD of quadruplicates.

Table 2.

Growth and adhesive properties of SP1, Cl-12-H and Cl-24-L cells

	SP1	Cl-12-H	Cl-24-L
Growth in 7% (plastic)	+++	+++	+++
Growth in serum-free medium	+	++	+
Adhesion to collagen type I	+	+++	-
Adhesion to fibronectin	+++	+++	+++
Adhesion to laminin	+	+++	+
Expression of $\alpha_2\beta_1$	+	++	-
Colony growth (7%)	++	+++	+

Legend:
Table 3 illustrates the properties of the parent SP1 cells and the SP1 cell clones, Cl-12-H and Cl-24-L in growth, colony and adhesion assays.

-	not detectable
+	marginal growth or adhesion
++	moderate growth or adhesion
+++	Maximal growth or adhesion

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Figure Legends

Figure 1. <u>Adhesion of SP1 cells to purified ECM proteins</u>

Linbro 96-well tissue culture plates were coated for 18 h at 4°C with BSA, FN, LM, VN, Coll I and Coll IV at concentrations ranging from 0.15 to 20 μg/ml (obtained by a two-fold serial dilution). The wells were washed with PBS and blocked with RPMI supplemented with 0.5 mg/ml BSA. SP1 cells in RPMI 1640 supplemented with 0.5 mg/ml BSA were added to each well (30,000 cells /well) in a 100 μl volume. After 45 min at 37°C, the unattached cells were removed by washing with warm PBS. The remaining cells were fixed with 3.7% paraformaldehyde and stained with a solution of 1% toluidine blue in a 1% solution of sodium borate. The absorbance was measured with an ELISA plate reader at a wavelength of 570 nm. Each point is expressed as the mean ± SD of triplicates. The experiment was repeated three times with similar results. The symbol designating each ECM protein is indicated in the figure legend at the top left hand corner of the graph.

Figure 2. <u>Morphology of SP1 cells on ECM proteins</u>

SP1 cells were harvested and washed once in RPMI 1640 supplemented with 0.5 mg/ml BSA. They were then seeded (50,000 cells/well) in Costar 24-well tissue culture plates (coated with the indicated ECM proteins at 10 μg/ml) and allowed to adhere. Nonadherent cells were not removed. After 45 min the cells were photographed using a Leica DM IL inverted microscope using technical pan film (ESTAR-AH-base). Magnification: 66X

SP1 cells on   (A) Fibronectin
                      (B) Vitronectin
                      (C) Collagen type I
                      (D) Laminin

Figure 3. <u>Analysis of SP1 integrin profile cultured on plastic and agar.</u>

SP1 cells were cultured on tissue culture plastic or as colonies on agar coated plates in RPMI 1640 medium supplemented with 7% FBS for 24 h. The cells were harvested and the number of cells normalized in each group, then surface labelled with 1.0 mCi ^{125}I . The cell pellets were lysed in RIPA buffer at 4°C for 30 min and the supernatants were precleared with rabbit anti-mouse IgG and protein A sepharose. The supernatents were aliquoted and incubated with anti-integrin antibodies and protein A sepharose. Immunoprecipitates were washed 4 times in lysis buffer and run on a 7.5% SDS-PAGE under non-reducing conditions. Antibodies used were; from left to right, polyclonal anti-β_1; polyclonal anti-human-α_2 (cytoplasmic); polyclonal anti-human-α_3 (cytoplasmic); polyclonal anti-human-α_5 (cytoplasmic); mouse monoclonal anti-α_6 (GoH3) and polyclonal anti-human-α_v (cytoplasmic). The gel was fixed in gel fixative (10% acetic acid, 40% methanol, 50% dH$_2$O) and dried at 80°C for 1.5 h and autoradiographed. A similar gel was repeated 4 times with similar results.

<u>Legend</u>:
"P"- cells grown on tissue culture plastic (adherent)
"A" - cells grown on agar (non-adherent)
NOTE: cytoplasmic indicates that the antibody is directed to the cytoplasmic domain of the integrin subunit.

Figure 4 <u>Effect of divalent cations on SP1 cell adhesion</u>

SP1 cells were harvested with 5 mM EDTA and washed once in PBS supplemented with 0.5 mg/ml BSA. The cells were resuspended in divalent cation-free PBS supplemented with 0.5 mg/ml BSA and seeded (30,000 cells/well) in Linbro 96-well tissue culture plates coated with BSA or FN (10 μg/ml) as described in the Materials and Methods. The medium was supplemented with Ca^{2+}, Mg^{2+}, or Mn^{2+} at the indicated concentrations. After 45 minutes, the non-adherent cells were removed by washing 4 times with warm PBS. The remaining cells were fixed with 3.7% paraformaldehyde and stained with 1% toluidine blue in 1% sodium borate. The absorbance was measured using an ELISA plate reader at 570 nm. The results are expressed as the mean ± SD of quadruplicates. The experiment was repeated twice with similar results.

Figure 5. <u>Adhesion assay in the presence of BMA5 antibody</u>

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Linbro 96 well tissue culture plates were precoated with fibronectin (10 μg/ml) or BSA (10μg/ml) overnight at 4°C. The wells were washed with PBS and blocked with RPMI 1640 supplemented with 0.5 mg/ml BSA for 2 h at 37°C. SP1 cells were seeded (30,000 cells/well) in the presence of no antibody (NT), GoH3 (hybridoma conditioned medium) (30% v/v), BMA5 antibody (hybridoma conditioned medium, 30% v/v) (BMA5 (sup)) and affinity purified BMA5 (10 μg/ml) (BMA5(conc)), or rabbit anti-mouse IgG (30 μg/ml) (IgG), After 30 min at 37°C the nonadherent cells were removed by washing with warm PBS. The remaining cells were fixed in 3.7% paraformaldehyde. The cells were stained in 1% toluidine blue and the absorbance measured in an ELISA plate reader at 570 nm. The results are expressed as the mean ± SD of triplicates. The experiment was repeated twice with similar results.

Figure 6. Effect of FN depletion on SP1 colony growth
        Serum was either untreated (UT) or incubated with protein A sepharose (-PAS) to remove endogenous IgG. After PAS treatment the serum was precleared with rabbit anti-mouse IgG (-IgG) and PAS. After preclearing the serum, fibronectin was removed by immunoprecipitation with a polyclonal anti-FN antibody (5μl/ml serum)(-FN). In one group bovine plasma fibronectin was added to the culture medium at a concentration of 10 μg/ml (-FN+FN). The colony assay was performed as described in the Materials and Methods in the presence of 7% FBS. The cells were incubated for 14 days then fixed in methanol and stained with Geimsa. The colonies were counted manually. The results are expressed as the mean ± SD of quadruplicates. The experiment was done 6 times and we observed an inhibition in 4 of the experiments (76%, 89%, 75% and 26%).

Figure 7. Effect of purified ECM proteins on SP1 colony growth
        The colony assay was performed as described in the Material and Methods. The purified matrix proteins indicated in the X axis were added at a concentration of 10 μg/ml to the RPMI 1640/agar mixture before solidification. All cultures contained 1% FBS. Inset: shows the effect of collagen type I on SP1 colonies in medium supplemented with 1% and 7% FBS. Colony growth was allowed to proceed for 14 days at 37°C. The colonies were fixed and stained as described in the Materials and Methods. The results are expressed as the mean ± SD of quadruplicates. The experiment was repeated four times. The colony assay with collagen was performed a total of 7 times. An inhibition of colony growth with Coll I was observed in 4 of the experiments (46%, 83%, 46% and 96%).

Figure 8. Incorporation of FITC-labelled FN in SP1 colonies
        SP1 colonies were grown on RPMI/agar coated plates supplemented with 3% FBS partially depleted of FN and 30 μg/ml of FITC-conjugated FN. After 5 days, the colonies were fixed in 3.7% paraformaldehyde, dried on glass slides, and viewed with a Meridian confocal microscope. (A) Phase contrast illumination; (B) fluorescence illumination. Magnification 1000X. The control, containing 30 μg/ml unlabelled FN, showed no immunofluorescence (data not shown).

Figure 9. Effect of HGF and TGF-β on SP1 colony growth
        The colony assay was performed as described in the Materials and Methods. The control group contained 1% FBS only, FN (10 μg/ml), HGF (5 ng/ml) and TGF-β (0.5 ng/ml) were added to the agar before solidification. The colonies were incubated for 14 days at 37°C. The colonies were fixed and stained as previously described. The experiment was performed in quadruplicate. The results are expressed as the mean ± SD of quadruplicates. The experiment was repeated 2 times with similar results.

Figure 10. Effect of TGF-β on SP1 cell adhesion.
        SP1 cells were cultured under (A) adherent or (B) anchorage-independent conditions (in agar coated plates) in the presence of 2% FBS and in the absence or presence of 0.5 ng/ml TGF-β for 24 h. SP1 cells were harvested and seeded (30,000/well) in 96-well plates precoated with BSA, FN, VN, LM, Coll I, and Coll IV at a concentration of 10 μg/ml as described in the Material and Methods. After 45 min at 37°C, the non adherent cells were removed by washing with warm PBS and the remaining cells were fixed and stained as previously described. The results are expressed as mean ± SD of triplicates.
Panel A: Adhesion assay of SP1 cells treated with TGF-β under adherent conditions
Panel B: Adhesion assay of SP1 cells treated with TGF-β under anchorage-independent conditions.

Figure 11. Effect of FN, HGF and TGF-β on SP1 cell survival

Panel A

   The survival assay was performed as described in the Materials and Methods. SP1 cells were seeded (20,000/plate) in 1.5 ml RPMI 1640 supplemented with 1% FBS in agar coated plates (35 x 10 mm) for 24 h at 37°C. The control group contains 1% FBS only. The treatment groups contain FN, HGF or TGF-β at the indicated concentrations in addition to 1% FBS. The reagents were added at the start of the experiment. The cells were then harvested in eppendoff tubes, spun down at 8000 rpm for 2 min and resuspended in 50 μl RPMI containing 4 μg/ml acridine orange and 4 μg/ml ethidium bromide. The cells were counted (at least 100/group) under a Leitz fluorescence microscope using an I filter. The experiment was repeated twice with similar results.

Panel B

The survival assay was performed as described in the Materials and Methods. The control group (solid bar) contains 1% FBS. The treatment groups contain 1% FBS as well as FN, Coll I or LM at a concentration of 10 μg/ml. The results are expressed as the mean $\pm$ SD of 3 experiments. * indicates a significant reduction in the percent survival compared to the control (p < 0.05).

Figure 12. SP1 cell survival in the presence of BMA5 antibody

        The survival assay was performed as described in the Materials and Methods. The control group contained 1% FBS. The treatment groups contain 1% FBS in addition to the indicated antibodies. BMA5 (hybridoma conditioned medium) was added at 30% v/v (sup). Affinity purified BMA5 (conc) was added at 10 μg/ml. Rabbit anti-mouse IgG (Rb αM IgG) and a non-blocking rat anti-α_5 mAb, 5H10 (anti-α5), were added at 30 μg/ml at the start of the assay. The cells were stained as previously described. The results are expressed as the mean $\pm$ SD of 3 experiments. * and ** indicates a significant reduction in the percent cell death as compared to the control (p < 0.05).

Figure 13. Effect of wortmannin on FN and HGF-mediated cell survival of SP1 cells

        SP1 cells were seeded (20,000/dish) in 1.5 ml RPMI 1640 with 1% FBS on agar coated plates (35 x 10 mm) for 24 h at 37°C. The medium was supplemented with FN (10 μg/ml) or HGF (10 ng/ml) as indicated. Wortmannin (Wort) was added to some groups at a concentration of 100 nM at the start of the experiment. After 24 h the cells were then harvested and stained with 4 μg/ml acridine orange and 4 μg/ml ethidium bromide. The cells (at least 100/group) were counted under a fluorescence microscope using an I filter. The results are expressed as the mean $\pm$ SD of four experiments. * indicates a significant reduction in the percent cell death as compared to the 1% FBS control (p < 0.05). ** indicates a significant reduction in the percent cell death as compared to the FN or HGF-treated groups (p < 0.05).

Figure 14. Effect of FN and HGF on cell survival on Δp85 transfectants

        Two SP1 cell clones transfected with Δp85, designated, C22 and C23, were seeded (20,000/dish) in 1.5 ml RPMI 1640 with 1% FBS on agar coated plates (35 x 10 mm) for 24 h at 37°C. Treatment groups contained FN (10 μg/ml) or HGF (10 ng/ml) or FN + HGF together. The cells were then harvested, stained and washed as described in the Materials and Methods. The results are expressed as the mean $\pm$ SD of four experiments. * indicates a significant reduction in the percent cell death as compared to the 1% FBS control (p < 0.05). ** indicates a significant reduction in the percent cell death as compared to the FN or HGF-treated groups (p < 0.05).

Figure 15. HGF receptor tyrosine phosphorylation under adherent and anchorage-independent conditions
Panel A

        SP1 cells were prestarved overnight in RPMI 1640 medium. Six h prior to harvesting some SP1 cells were harvested and plated on non-tissue culture plastic coated with BSA. Prior to harvest (30 min) some groups were treated with 20 ng/ml recombinant HGF. Cells were harvested using 5 mM EDTA and lysed in 1% NP40 lysis buffer, immunoprecipitated with anti-c-Met, analyzed by 6.0% SDS-PAGE and transferred to nitrocellulose followed by western blotting. The blot was immunoblotted with anti-phosphotyrosine antibody and the bands visualized using ECL reagents. The experiment was repeated twice with similar results.

Panel B. The same blot as in panel A was stripped (100 mM TRIS, 4% SDS, at 50°C for 30 min) and reprobed with anti-c-Met. The proteins were visualized using ECL reagents.

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Figure 16. <u>Western blot analysis of FAK phosphorylation under adherent and anchorage-independent conditions</u>

SP1 cells were prestarved in RPMI 1640 for 24 h, harvested and replated on Poly-L-lysine (PLL) (1:10 dilution) or FN-coated plates (10 μg/ml) for adherent groups (FN). One adherent group was also treated with HGF (20 ng/ml) (FN + HGF). The anchorage-independent (AI) groups were incubated in 10 ml tubes with FN (40 μg/ml) or HGF (20 ng/ml) or a combination of both for 30 min at 37°C. The cells were lysed in 1% NP-40 lysis buffer and the total protein concentration standardized in each group. FAK was immunoprecipitated with anti-FAK mAb. The immunoprecipitates were washed in lysis buffer and resolved by 8.0% SDS-PAGE. The proteins were transferred to nitrocellulose and blotted with anti-phosphotyrosine antibody then, visualized by ECL.

Figure 17. <u>Western blot analysis of PI 3-kinase phosphorylation under adherent and anchorage-independent conditions</u>

SP1 cells were prestarved for 24 h, harvested and replated on Poly-L-lysine(PLL) or FN-coated plates (FN). One adherent group on FN was treated with HGF at 20 ng/ml (FN + HGF) The anchorage-independent (AI) groups were incubated in 10 ml tubes in the presence of FN (40 μg/ml) or HGF (20 ng/ml) for 60 min at 37°C. The cells were lysed in 1% NP-40 lysis buffer and PI 3-kinase was immunoprecipitated with anti-PI 3-kinase rabbit IgG. The immunoprecipitates were washed in lysis buffer and resolved by 8.0% SDS-PAGE under reducing conditions. The proteins were transferred to nitrocellulose and blotted with anti-phosphotyrosine antibody and visualized by ECL. The experiment was performed twice with similar results. Panel A: blotted with anti-phosphotyrosine antibody Panel B: blotted with anti PI 3-kinase

Figure 18. <u>PI 3-kinase activity in response to HGF and FN under adherent and anchorage-independent conditions</u>

SP1 cells were maintained anchorage-independently for 1 h at 37°C in the absence or presence of HGF (20 ng/ml) or FN (20 μg/ml) or both (FN+HGF) or 7% FBS. The control group is a sham group, which contains no immunoprecipitated proteins The cells were lysed in 1% NP40 lysis buffer and PI 3-kinase immunoprecipitated with anti-phosphotyrosine antibody. The immunoprecipitates were washed and incubated with the substrate phosphatidylinositol and 15 μCi gamma ^{32}P-ATP. After 20 min, the reaction was stopped with 6 M HCl and the lipids were extracted with chloroform/methanol and resolved by TLC. The plate was autoradiographed and the radioactive area corresponding to the radiolabelled phosphoinositol 3-phosphate (PI 3-P) was removed and measured in a scintillation counter.

Figure 19. <u>Morphology of Cl-12-H and Cl-24-L on plastic</u>

Cl-12-H and Cl-24-L cells were seeded in 100 x 15 mm tissue culture plates in the presence of 7% FBS. After 8 h, the cells were photographed using a Leica DM IL inverted microscope using Kodak technical pan film (ESTAR-AH base). Magnification: 130 X
A: Cl-12-H
B: Cl-24-L

Figure 20. <u>Growth of SP1, Cl-12-H and Cl-24-L cells on plastic in serum-free conditions and 7% FBS</u>

SP1 (empty bars), Cl-12-H (solid bars) and Cl-24-L cells (hached bars) were seeded (10,000/well) in 24 well tissue culture plates in the test medium (7% FBS or serum-free). After 24 h, 0.2 μCi ^{3}H-thymidine was added to each well and incubated for another 24 h at 37°C. The cells were harvested with trypsin-EDTA and placed in 96-well microtiter plates then transferred to filters using a cell harvester. ^{3}H-thymidine incorporation was measured (CPM/well) in a scintillation counter using Ecolume scintillation fluid. The results are expressed as the mean ± SD of four experiments; each experiment contained quadruplicate samples. ✱ indicates a significant increase in growth compared to SP1 and Cl-24-L cells grown under serum-free conditions ($p<0.05$).

Figure 21. <u>Adhesion of Cl-12-H and Cl-24-L cells to collagen</u>
Panel A

The cell adhesion assay was performed as described in the Materials and Methods. Linbro 96-well tissue culture plates were precoated with collagen types I and IV coated at the concentrations indicated (obtained by two-fold serial dilution). After 45 min at 37°C the cells were fixed and stained as described in the Materials and Methods. The results are expressed as the mean of duplicate samples. The experiment was repeated twice with similar results.

37

Panel B and C. <u>Photographs of Cl-12-H and Cl-24-L on collagen type I</u>
        SP1 cells were harvested and seeded in Linbro 96-well tissue culture plates (30,000 cells/well coated with collagen type I (10 µg/ml). The cells were allowed to adhere for 30 min then photographed on a Lieca DM IL inverted microscope using technical pan film. Magnification: 120 X
B: Cl-12-H on collagen type I
C: Cl-24-L on collagen type I

Figure 22. <u>Collagen receptors on Cl-12-H and Cl-24-L cells</u>
Panel A
        Cl-12-H and Cl-24-L cells were harvested and labelled with a biotinylation reagent to biotinylate all cell surface proteins in PBS as described in the Materials and Methods. The cells were counted and the cell number normalized in each group then lysed in RIPA lysis buffer. The lysate were centrifuged and the protein concentration of the supernatant normalized and aliquoted into 4 groups. The integrins were immunoprecipitated with rabbit anti-mouse IgG (control), rabbit anti-β_1, anti-α_2 and anti- α_3 IgG and resolved by 7.5% SDS-PAGE under non-reducing conditions. The proteins were transferred to nitrocellulose and a western blot analysis performed using a streptavidin-horse radish peroxidase-conjugated antibody for detection. The proteins were visualized using ECL reagents. The vertical arrows ↓ show the position of the α_2 and α_3 subunits of Cl-12-H cells.

Panel B
An aliquot of the same immunoprecipitate as in Figure 18A was run under reduced conditions (5% 2-mercaptoethanol) and resolved by 7.0% SDS-PAGE. The proteins were transferred to nitrocellulose and a western blot analysis performed using a streptavidin-horse radish peroxidase-conjugated antibody for detection. The proteins were visualized using ECL reagents.

Figure 23. <u>Colony growth of Cl-12-H cells in the presence of exogenous ECM proteins</u>
Panel A
        The colony assay was performed as described in the Materials and Methods. The control group contains 1% FBS. The treatment groups also contain ECM proteins indicated on the X axis at a final concentration of 10 µg/ml before solidification. After 14 days, the plates were fixed and stained as previously described. The results are expressed as the mean ± SD of quadruplicates.

Panel B
        The colony assay was performed as described in the Materials and Methods. SP1, Cl-12-H and Cl-24-L cells were seeded (2000/dish) in the presence of 7% FBS. Treatment groups also contained FN or collagen type I at 2.5, 5, or 10 µg/ml.

Figure 24. <u>Hypothetical model for the role of growth factors and ECM in a stepwise progression for colony growth.</u>

Adherent transformed cells progressively lose the requirement for adhesion. Once in an anchorage-independent state, soluble extracellular matrix proteins (ECM) bind to integrin receptors on the cell surface (P9) and growth factors (GF) bind to their respective receptors (Y) on the cell surface. These interactions provide survival signals to the cell. The cell then can slowly start to proliferate and organize ECM on its cell surface. Once several cells have clustered together, there is formation of ECM fibrils and a change in cell shape which provides the adhesive signals required to proliferate more rapidly and form a three-dimensional spheroid.

Figure 1
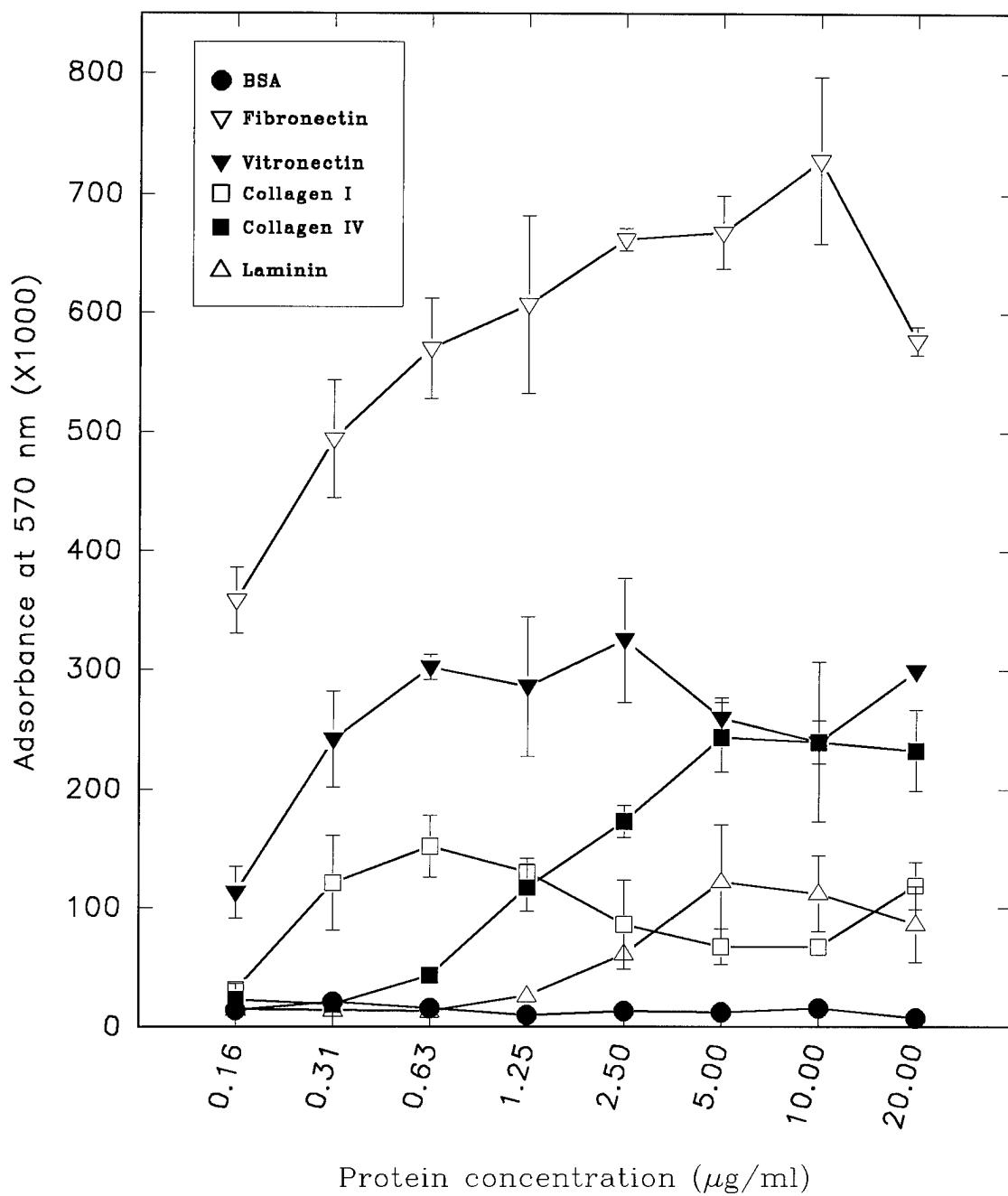
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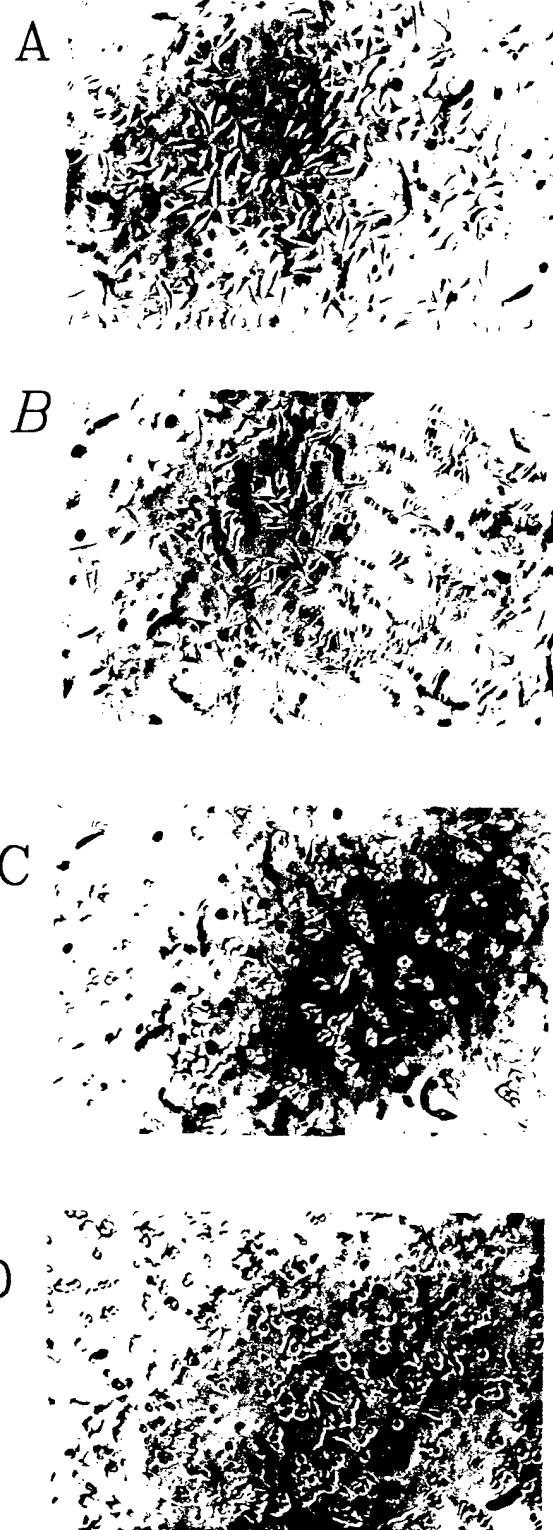
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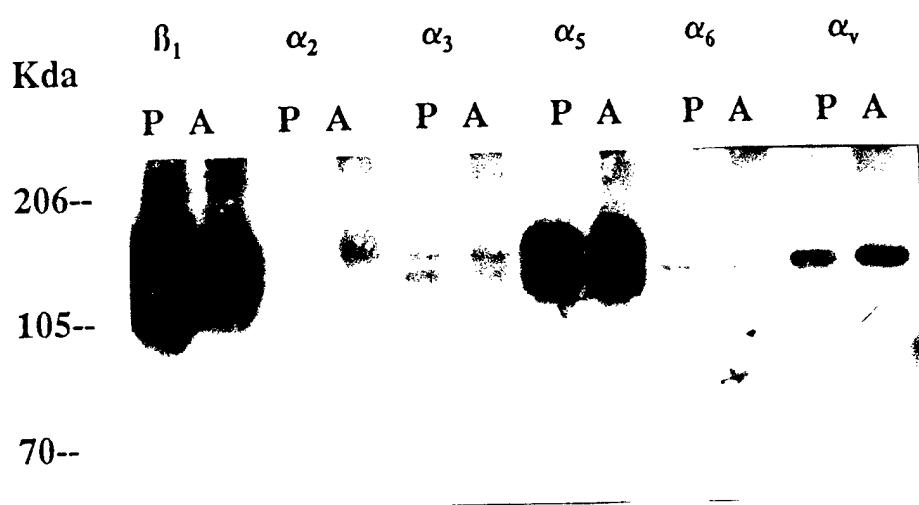
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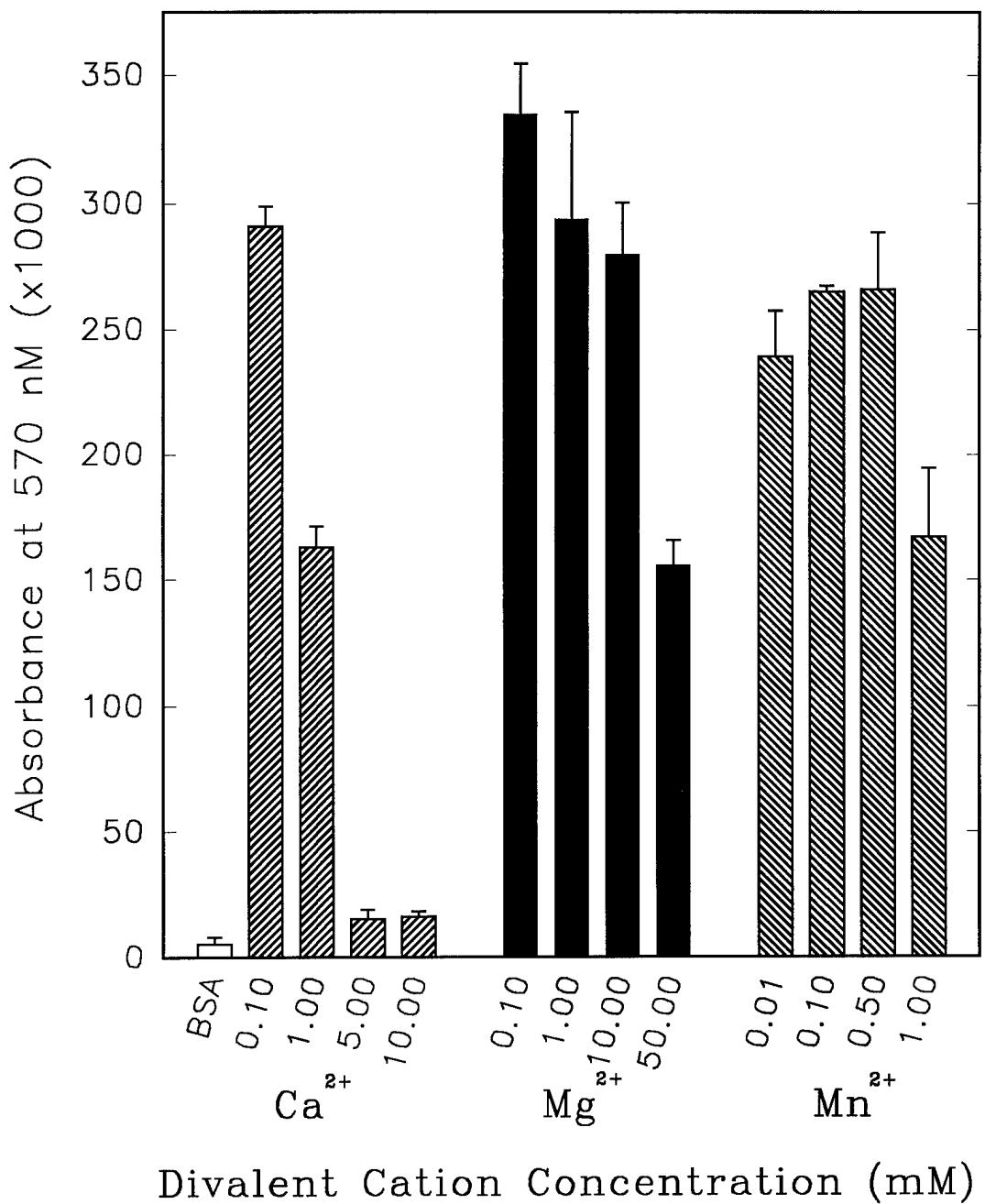
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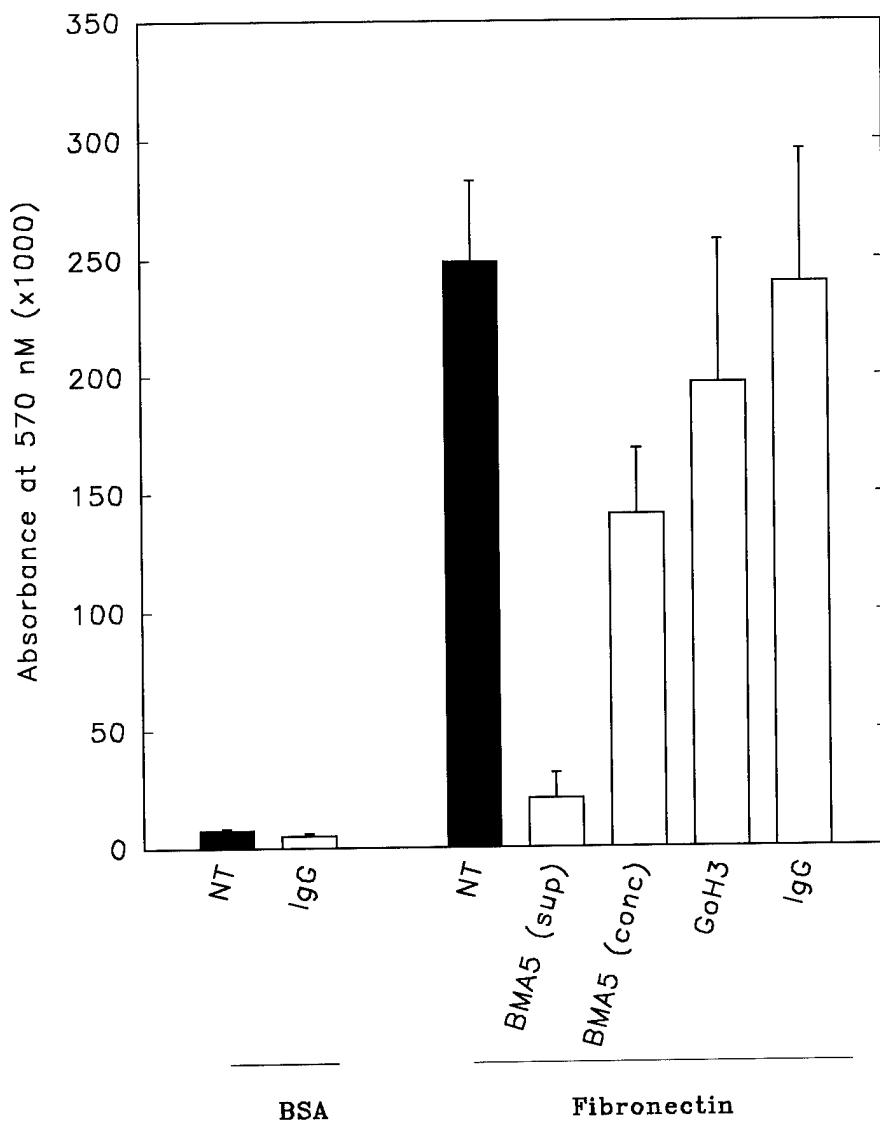
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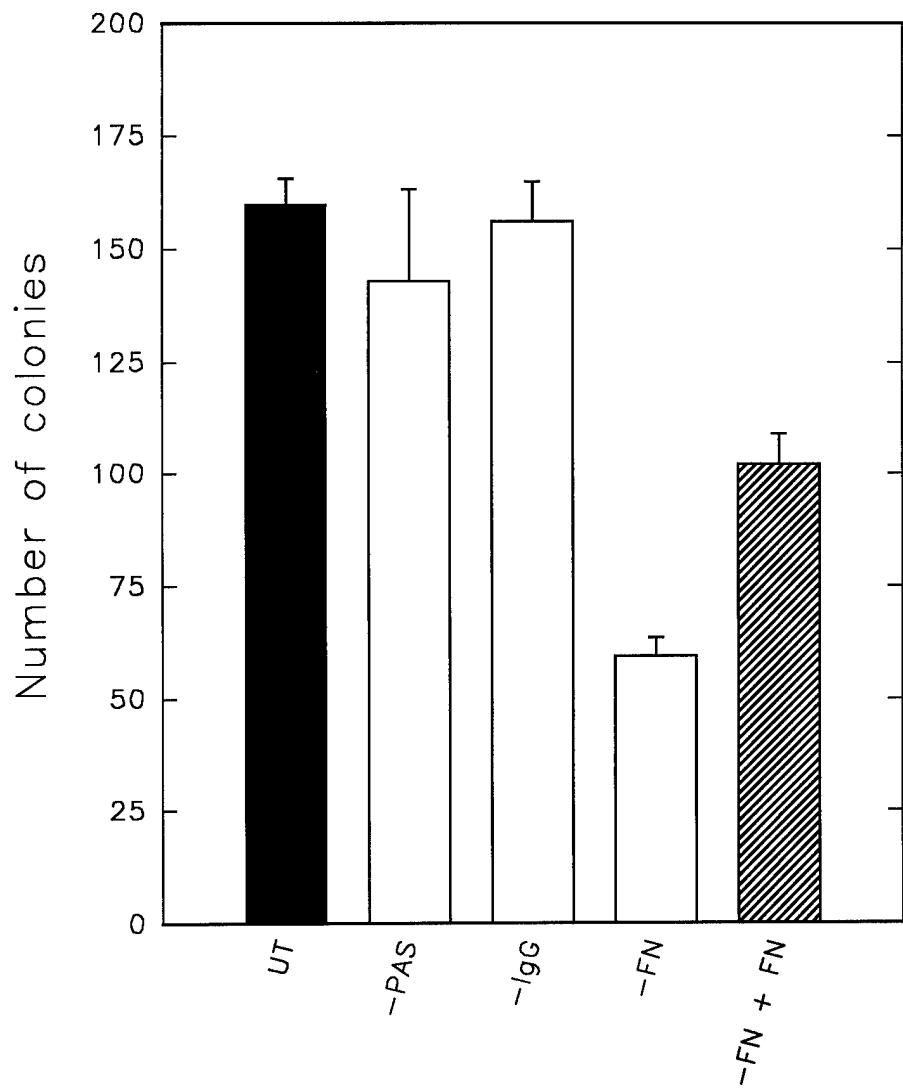
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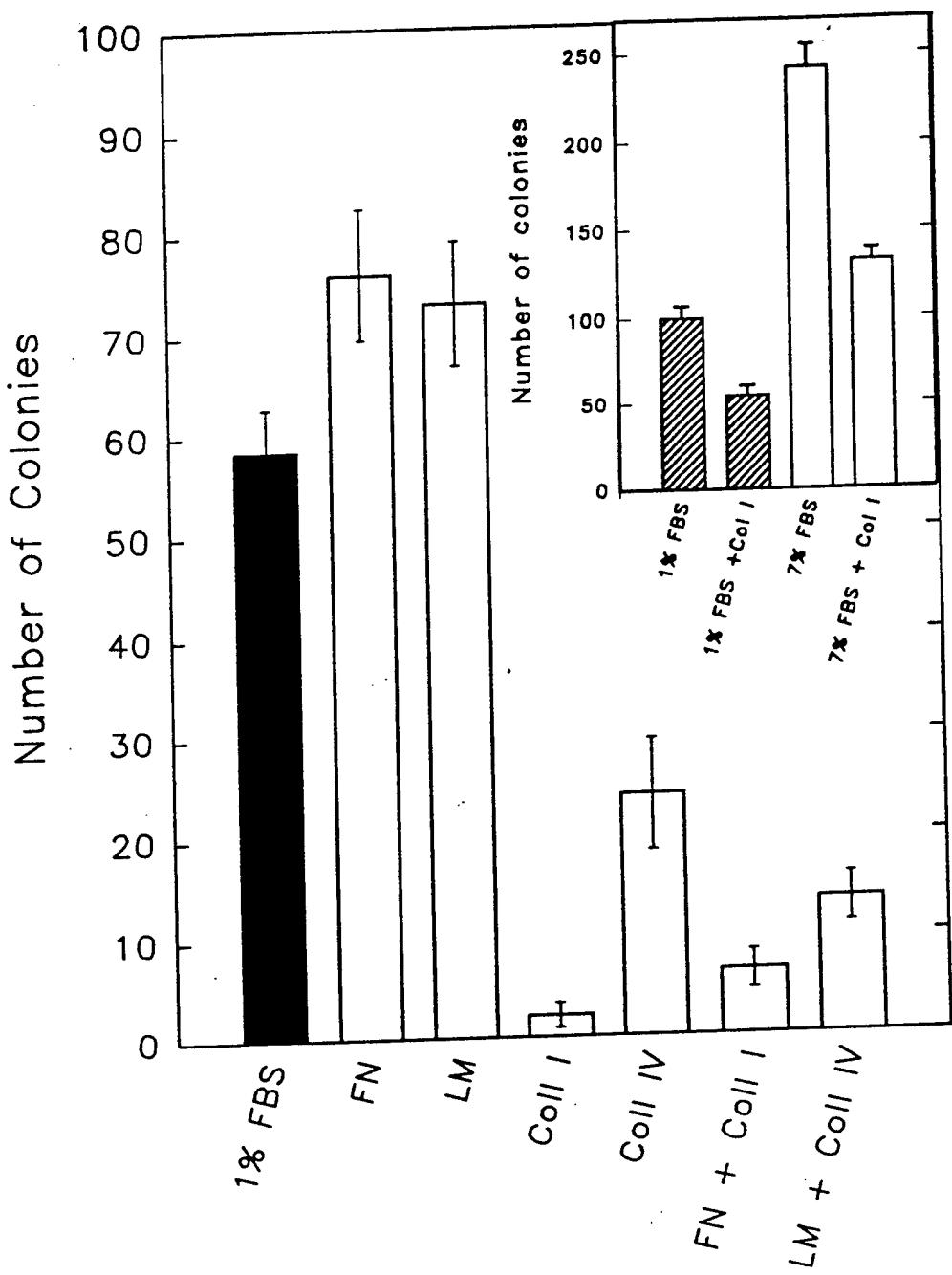
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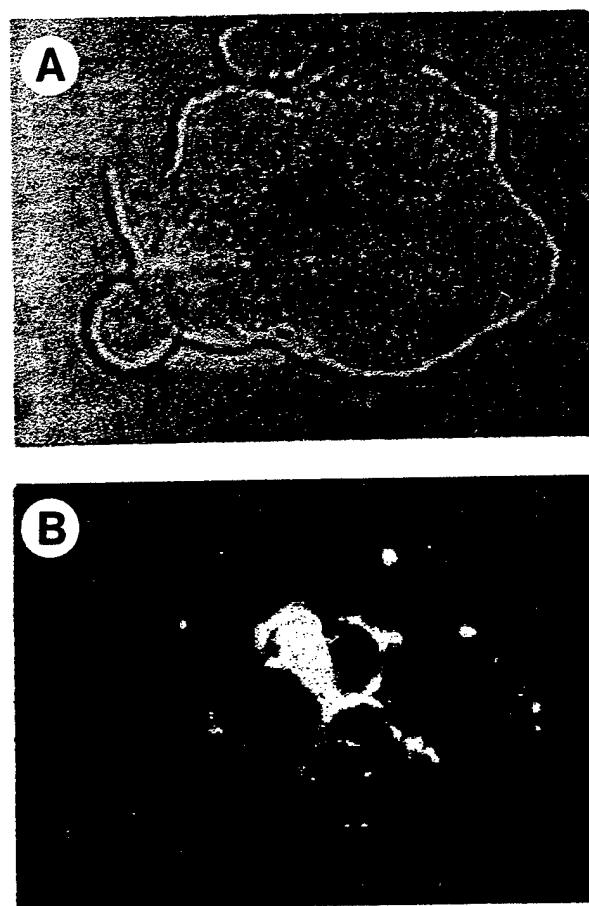
Figure 9

# Figure 10

A

B

48

Figure 11

A

B

49
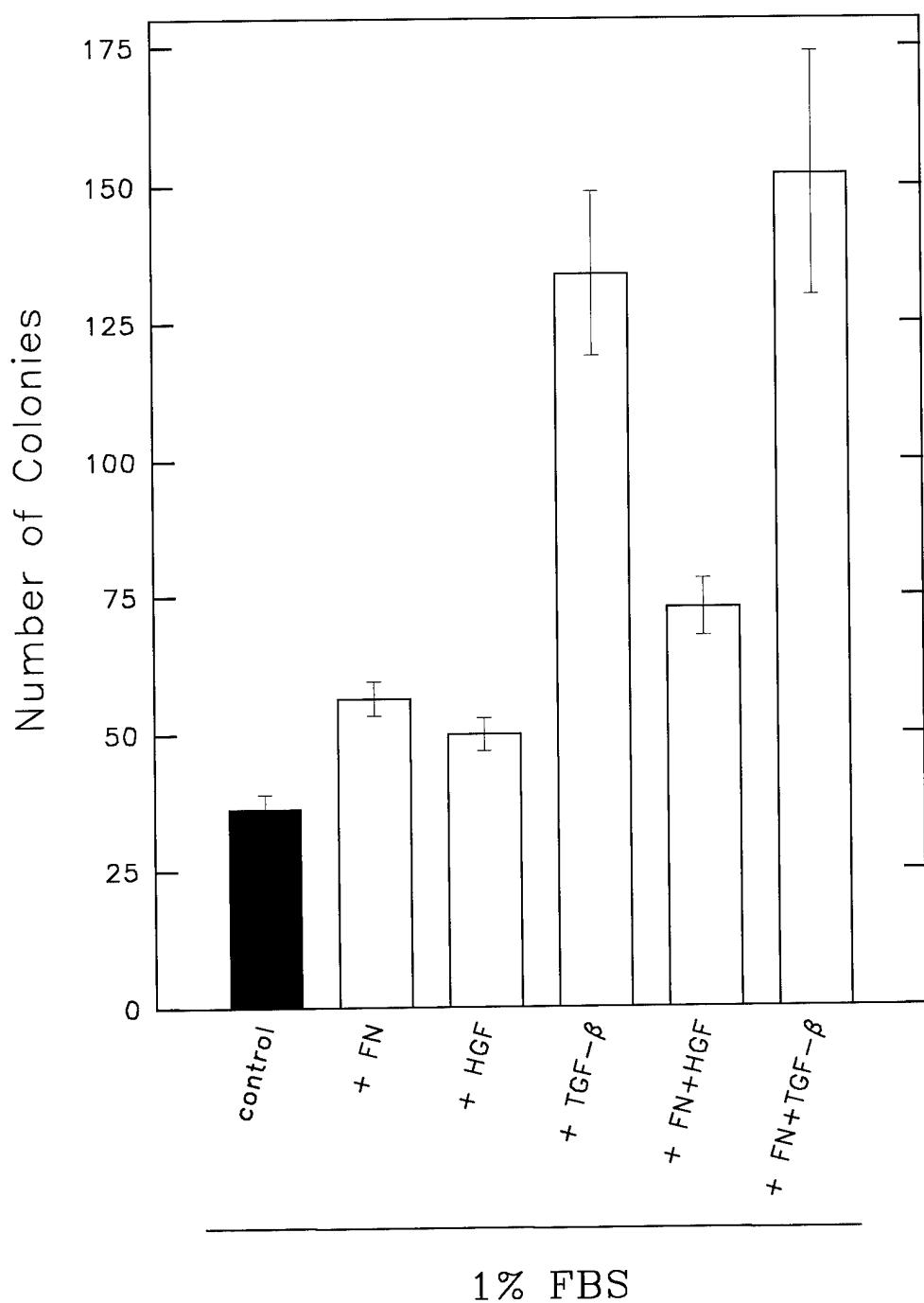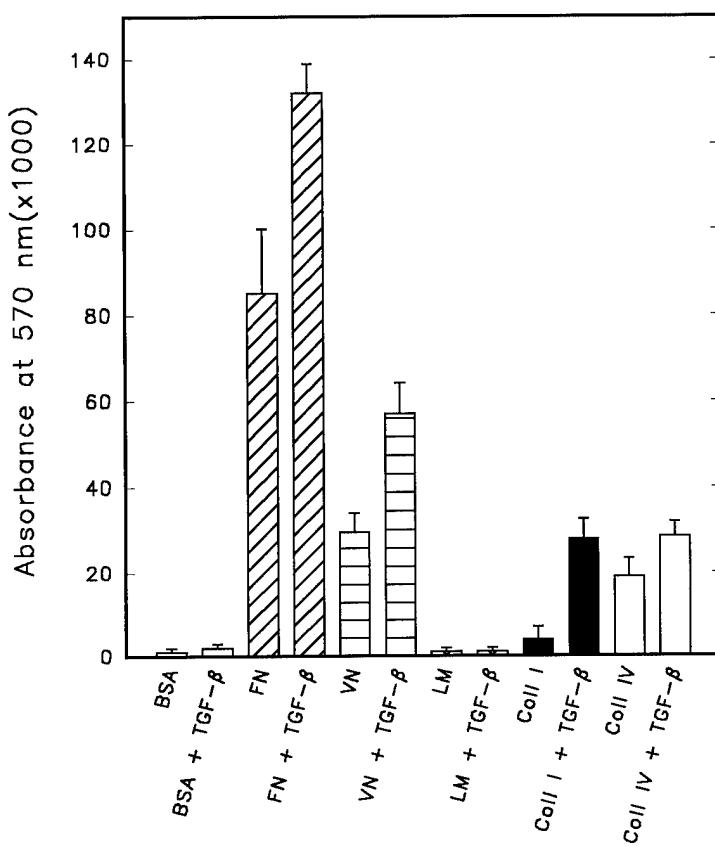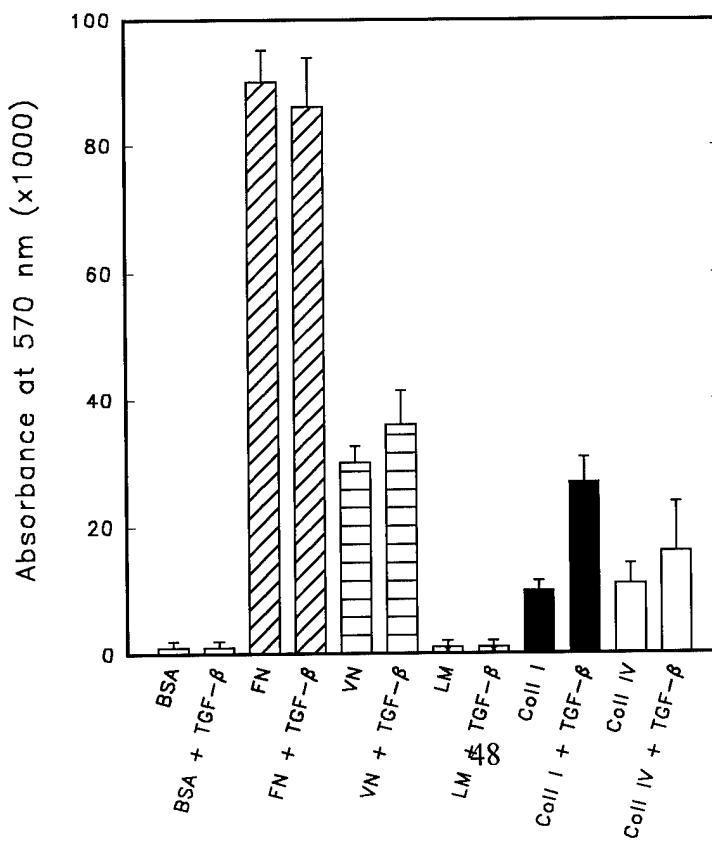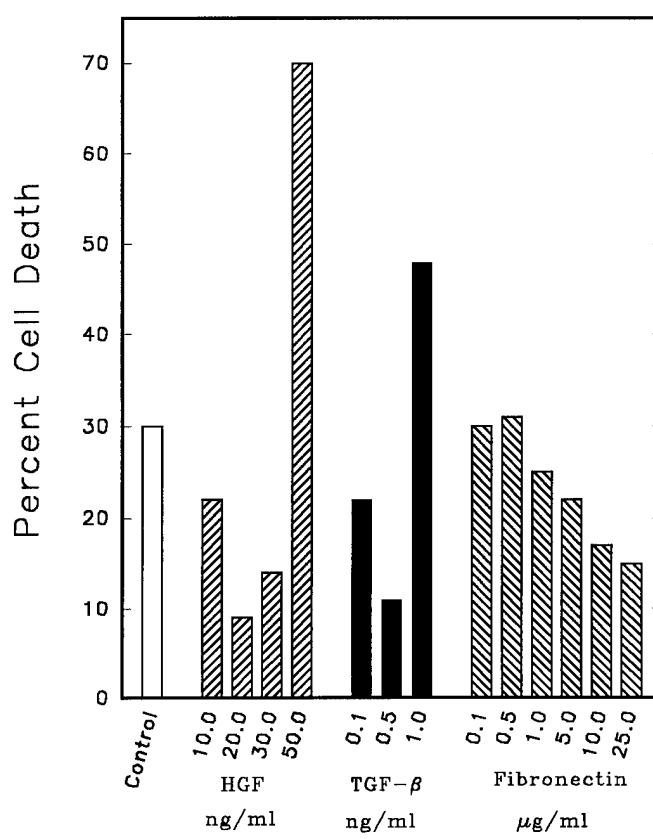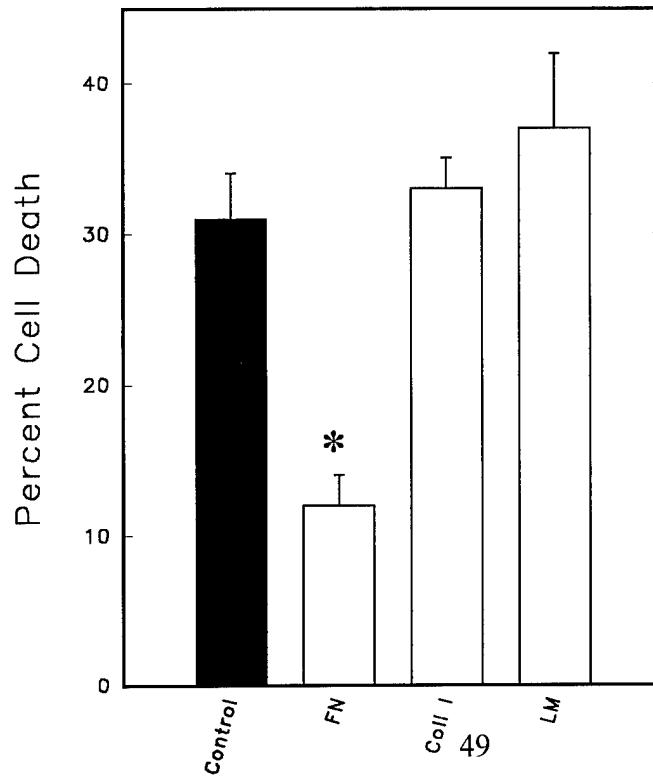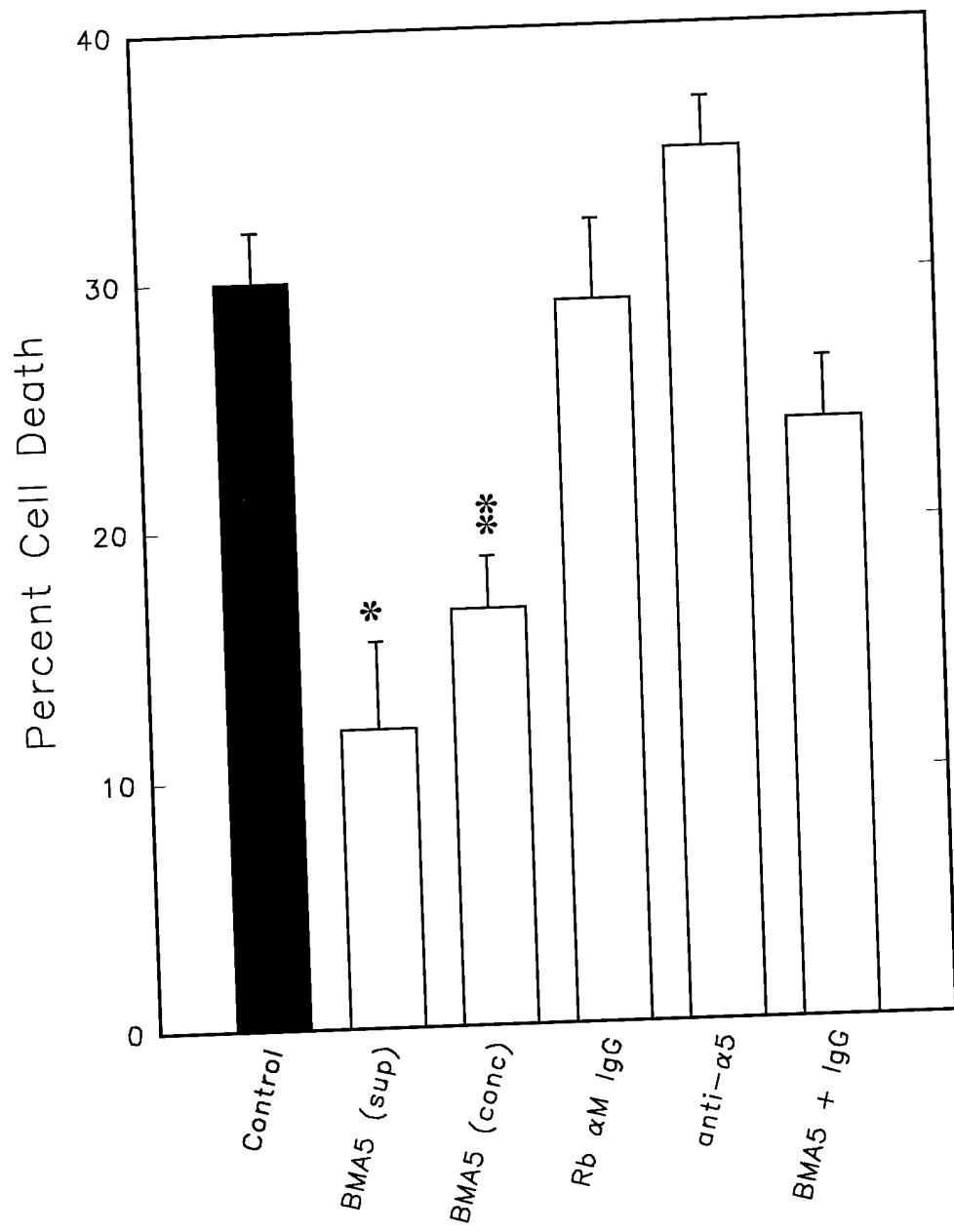
Figure 12

Figure 13

Figure 14

Figure 15

A

Non - Adherent

Adherent

Control HGF | Control HGF

KDa
- 214

p145^met →

- 111

- 74

IP: α-met
Blot: α-PY

B

Non - Adherent

Adherent

Control HGF | Control HGF

KDa
- 214

p145^met →

- 111

- 74

IP: α - met
Blot: α-met

Figure 16

p125 →

IP: anti-FAK
Blot: anti-PY

Figure 17

A

Adherent   Anchorage-independent

p85 →

IP: Anti-PI 3-kinase
Blot: Anti-PY

B

p85 →

IP: Anti PI 3-kinase
Blot: Anti PI 3-kinase
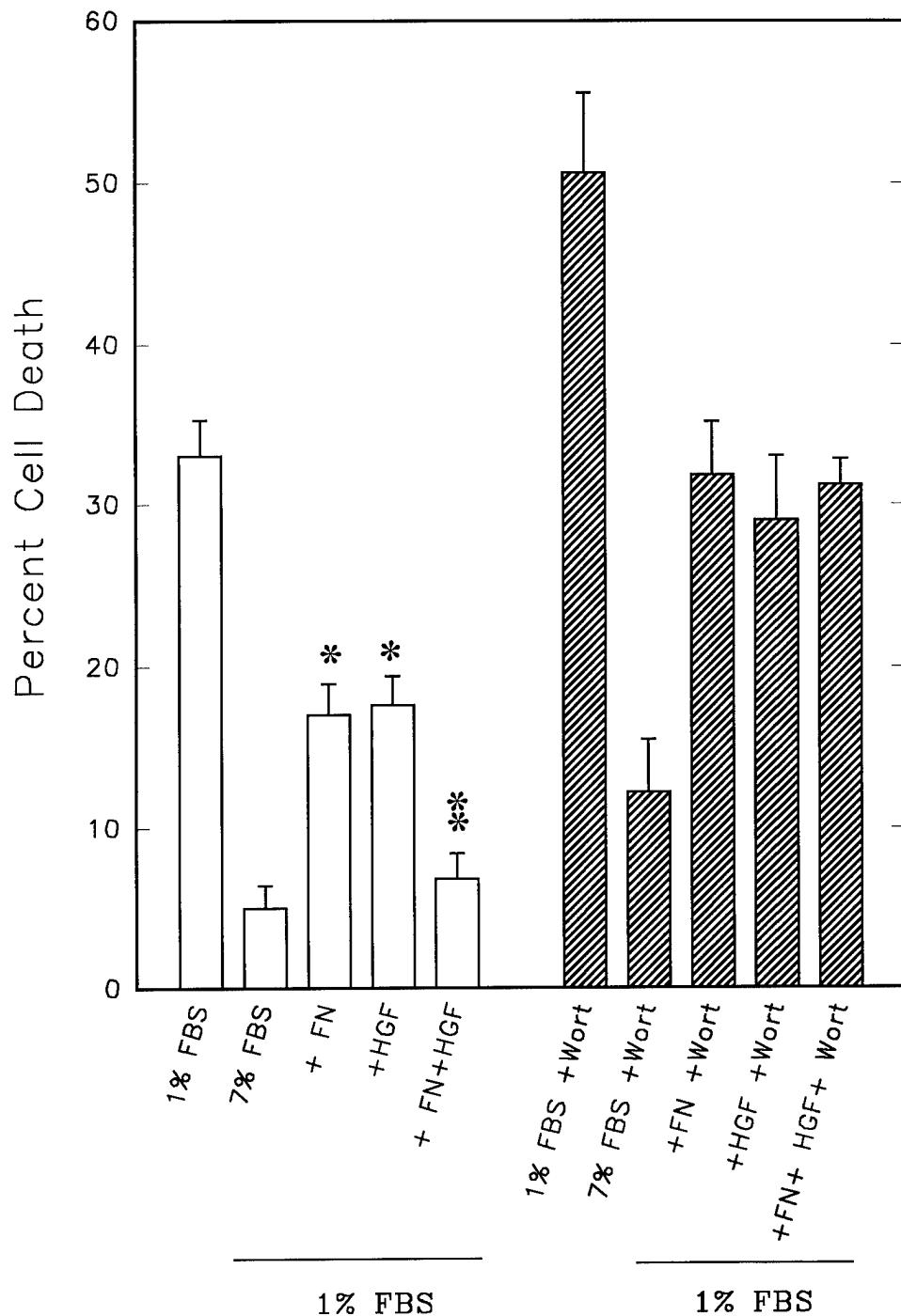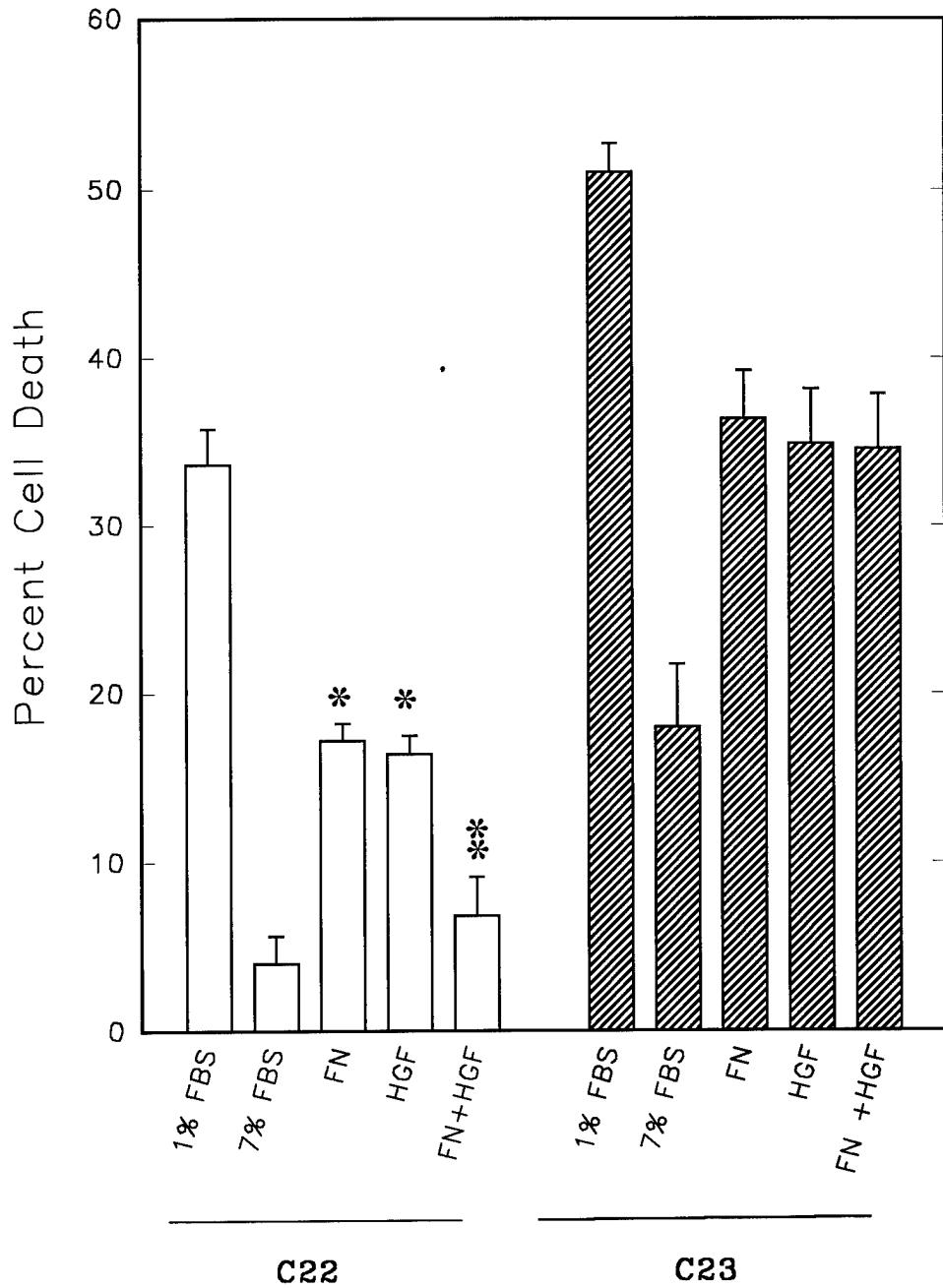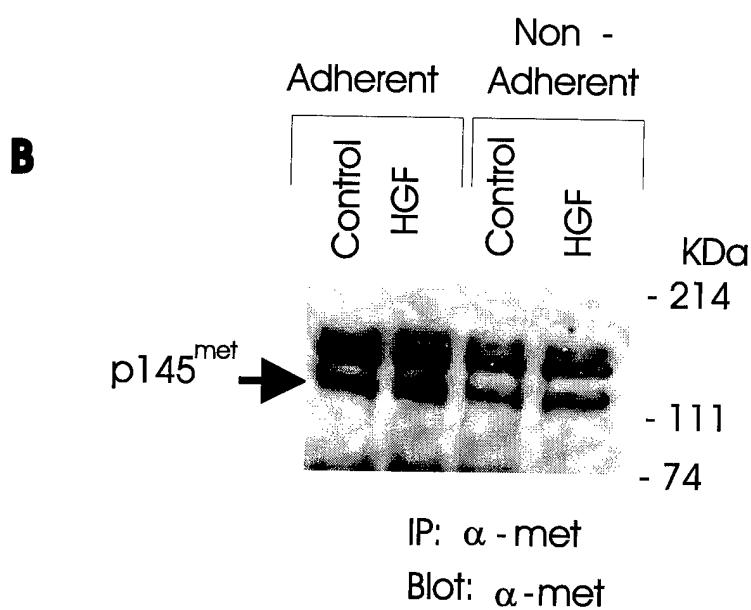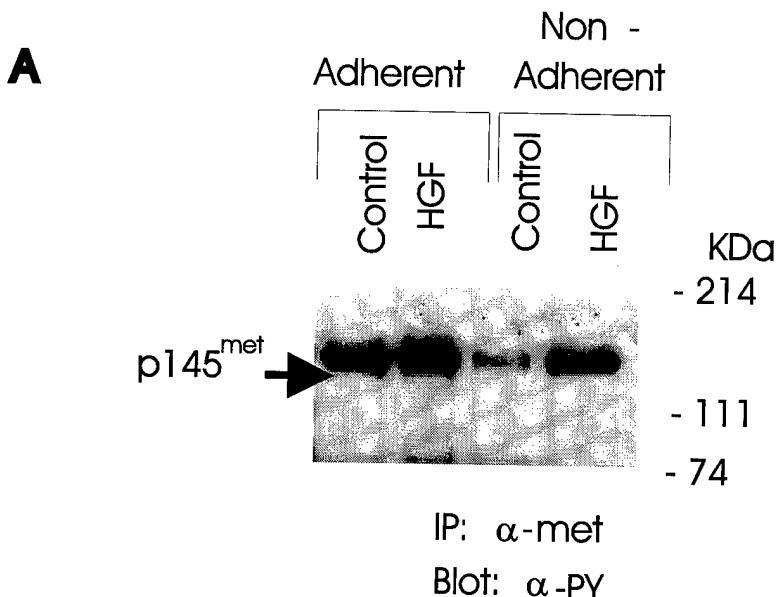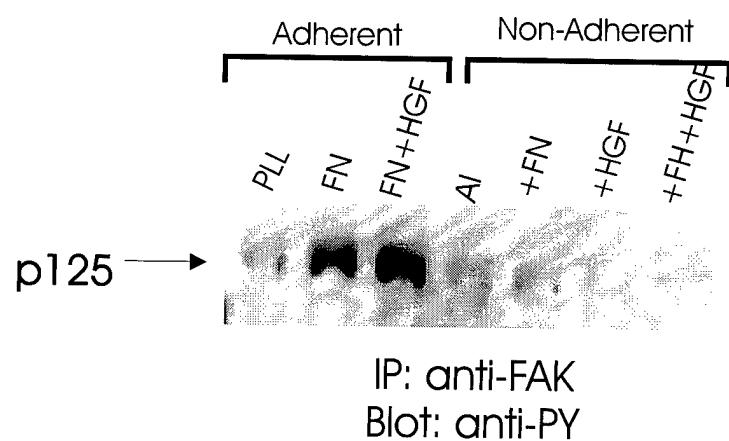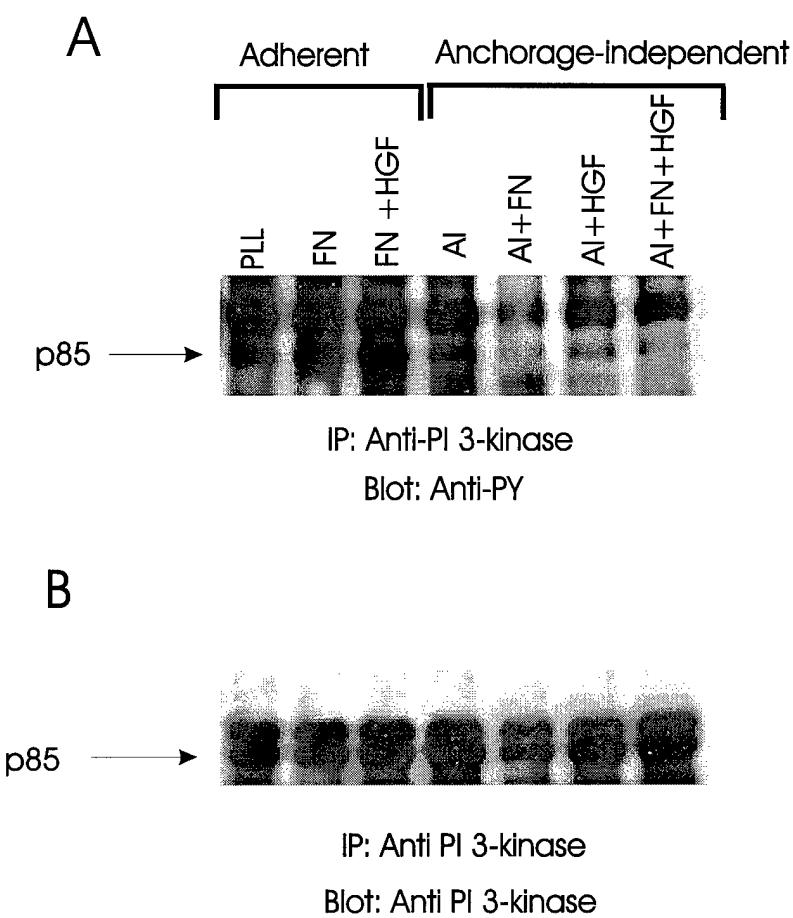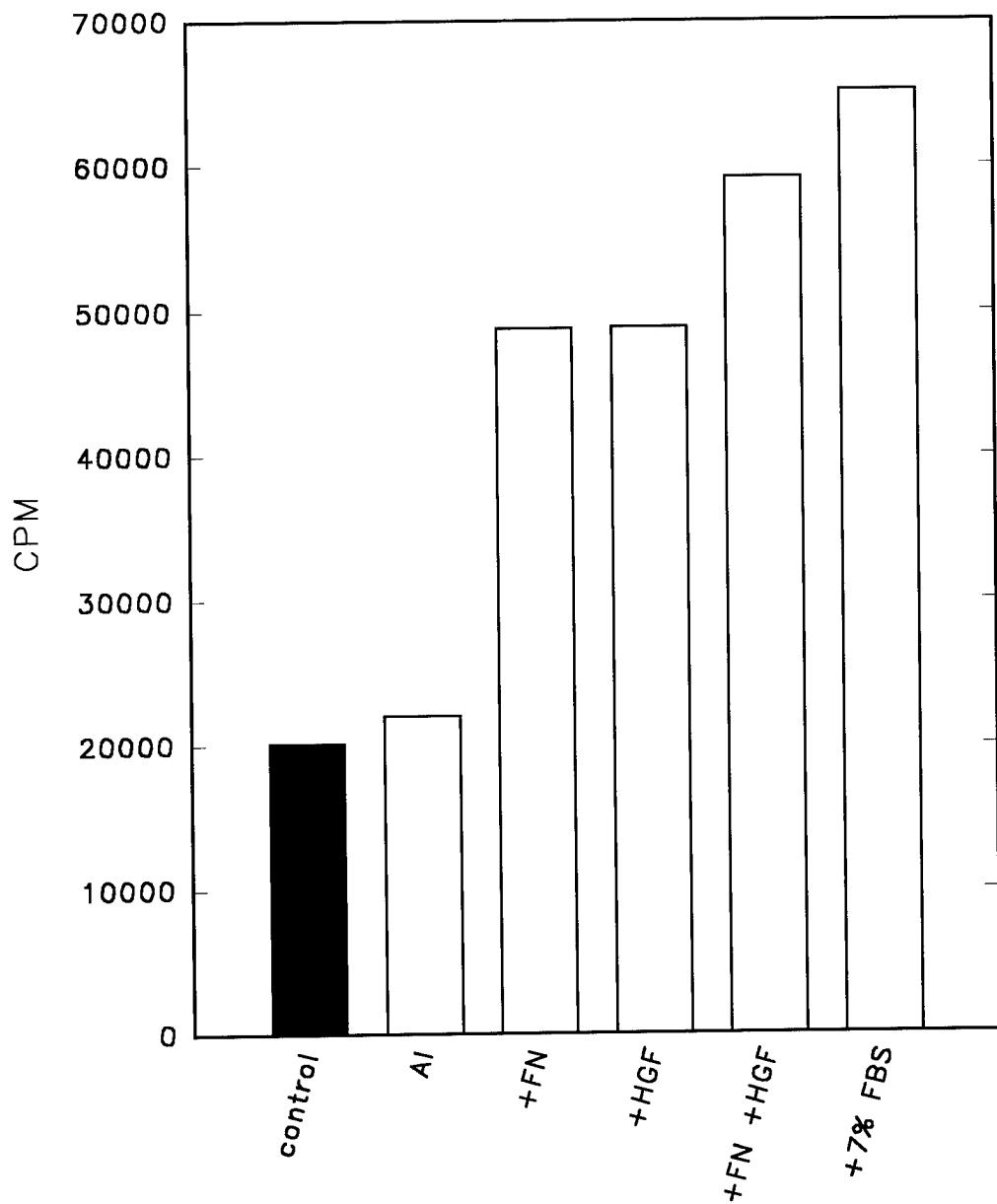
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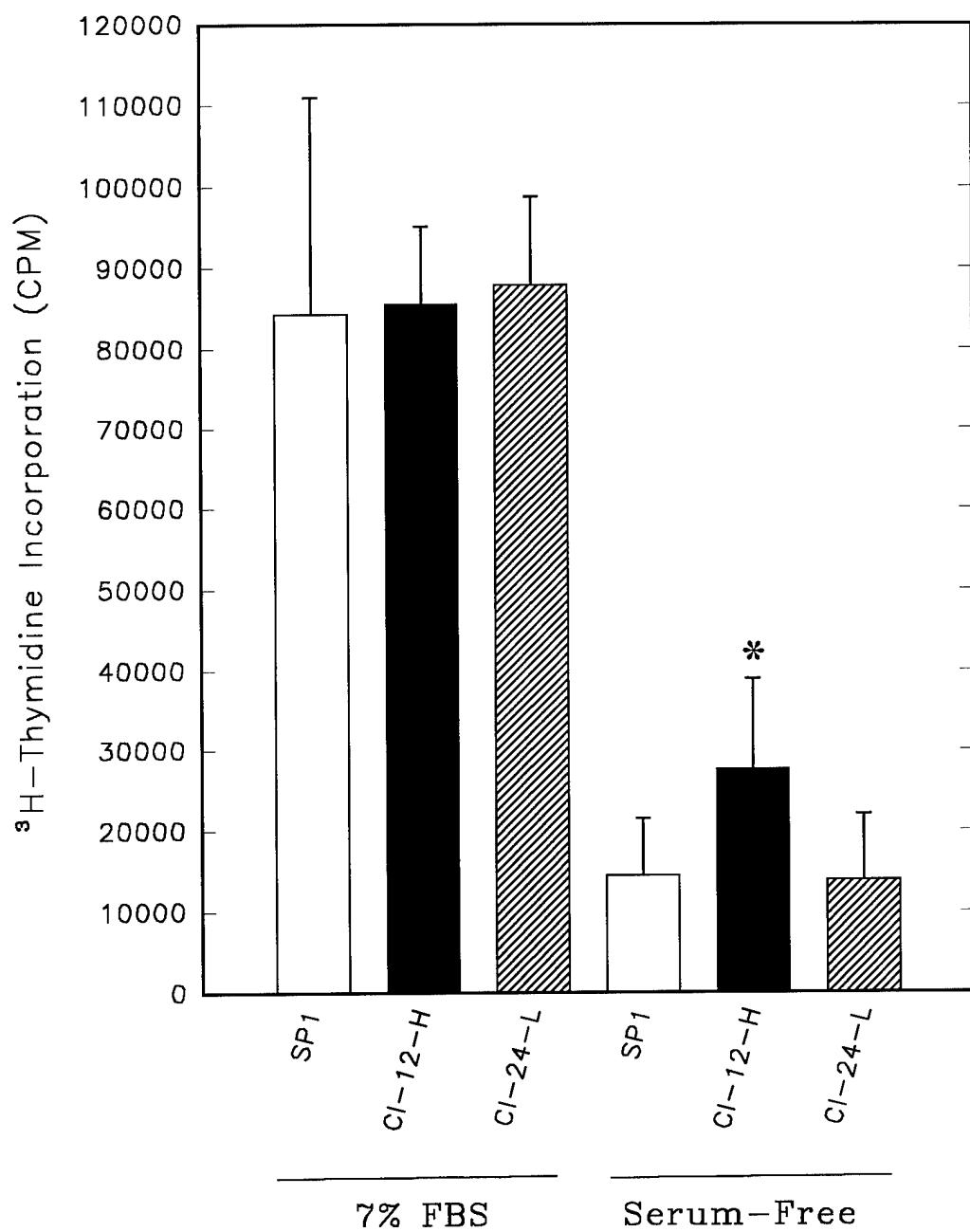
Figure 19

Figure 20

A
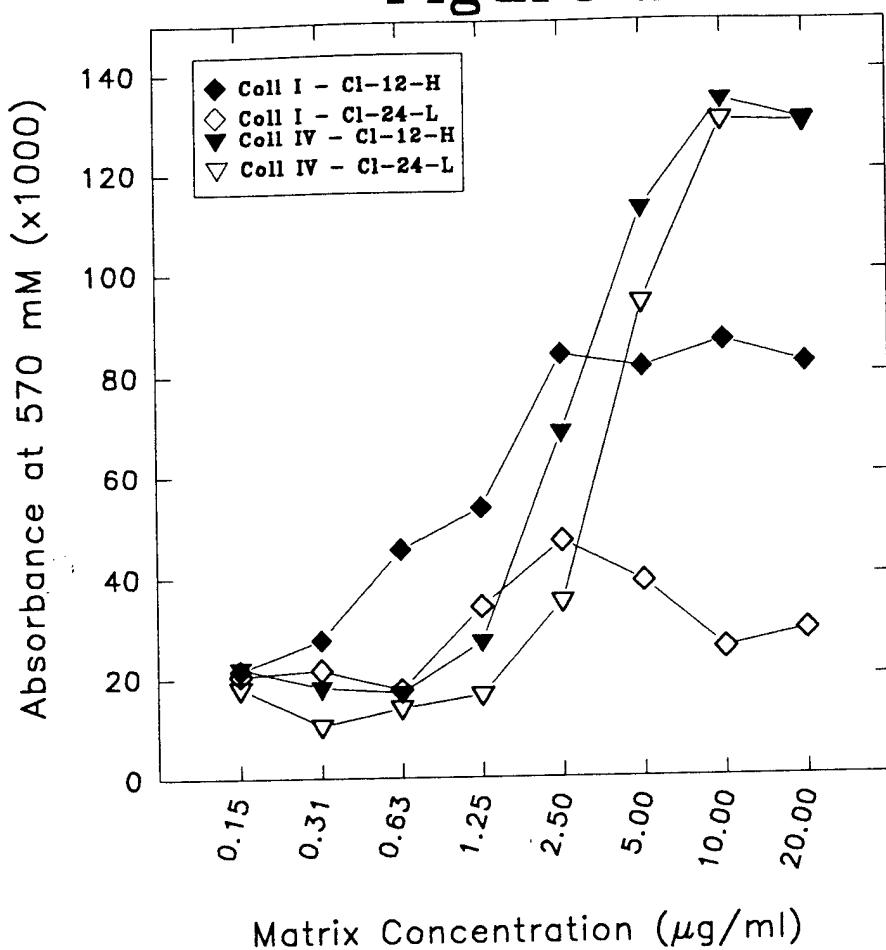
Figure 21

B

C
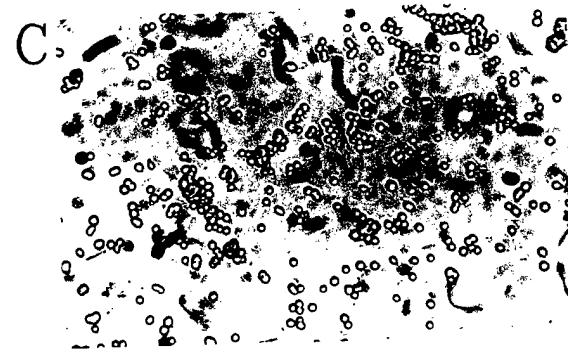
Figure 22

A

B

Figure 23

A

B

61
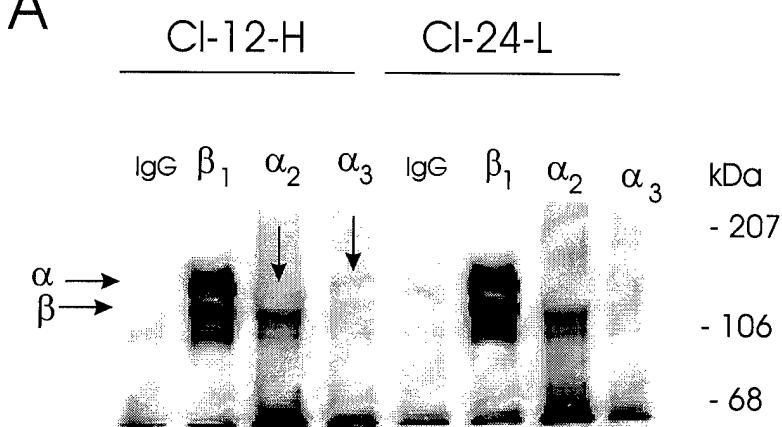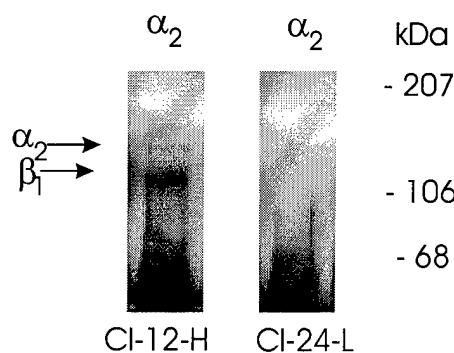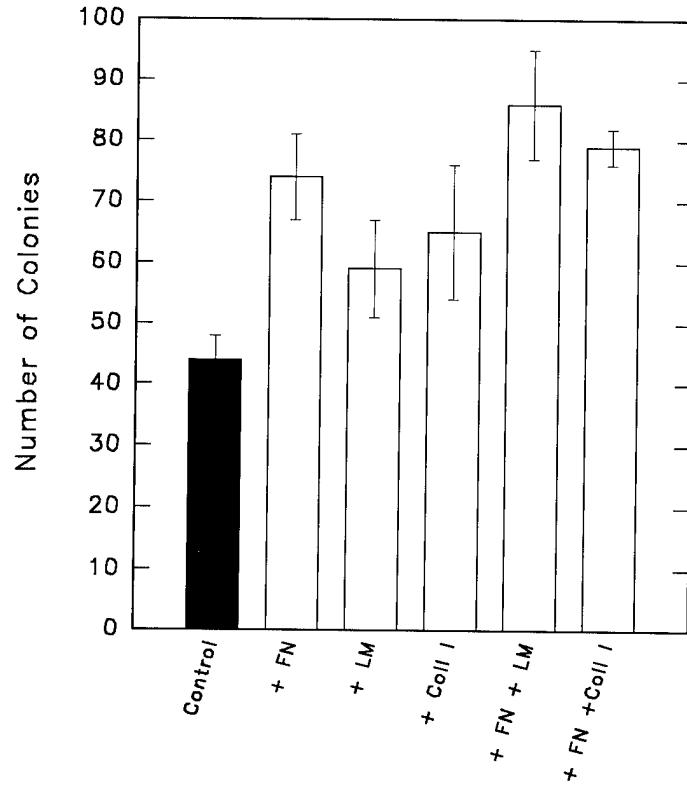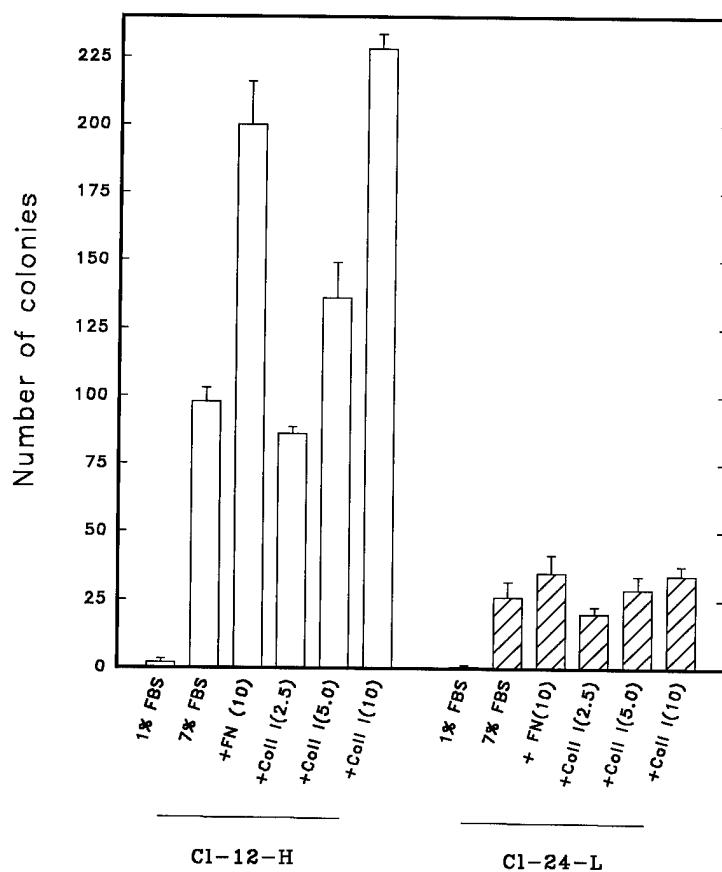
Figure 24

Stepwise progression for colony formation

Adherent transformed cell

GF

ECM, survival, proliferation

Loss of adhesion requirement

ECM GF Survival factors required

Slow Proliferation

Cell shape changes

GF GF

Matrix fibrils provide structure

AI growth rapid proliferation

GF ECM

Tumor spheroid

GF ? ECM ?
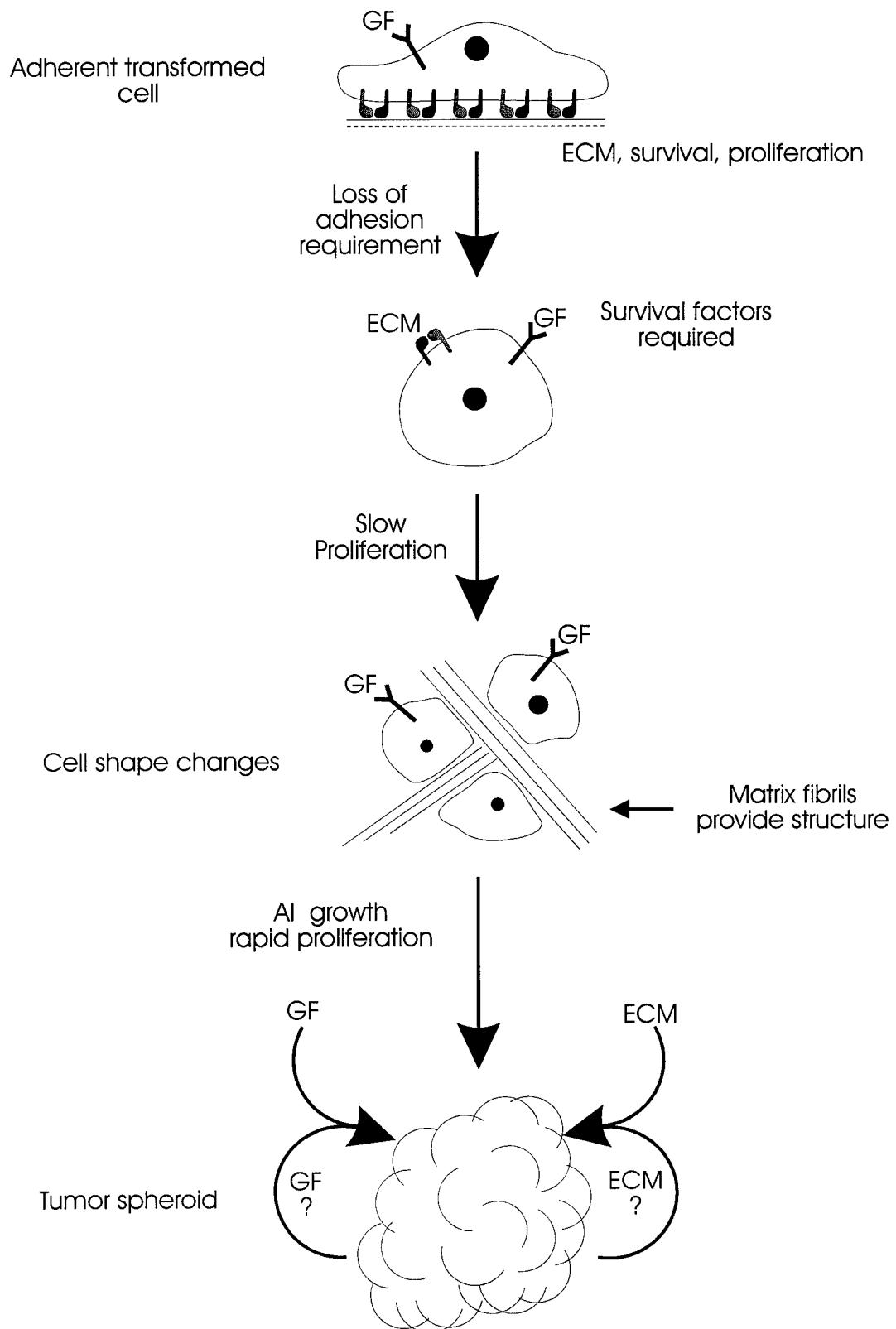
List of publications

1. Saulnier,R., B. Bhardwaj, J. Klassen, D. Leopold, N. Rahimi, E. Tremblay D. Mosher and B.E. Elliott. Fibronectin fibrils and growth factors stimulate anchorage-independent growth of a murine mammary carcinoma. Experimental Cell Research. 222:360-369, 1996.

2   Saulnier,R., S. Uniyal, T. Chau, A. Patrzykat, B.M.C. Chan, and B.E. Elliott. The role of integrins and extracellular matrix in anchorage-independent growth of a mammary carcinoma cell line. Cell. Mol. Biol. 43(3):455-468, 1997.

3. Saulnier,R., A. Patrzykat, P. Shenoy, and B.E. Elliott HGF and fibronectin mediated cell survival under anchorage-independent conditions: A role for PI 3-kinase (manuscript in preparation)

List of meeting abstracts

1. Elliott, B.E., B. Bhardwaj, R. Lall, D. Leopold, L. Maxwell, R. Saulnier, E. Tremblay, and K. Rubin. 3T3L1 adipocytes stimulate anchorage-independent growth of a murine mammary carcinoma. Possible role of fibronectin. UCLA Meeting on "Extracellular Matrix in Development and Disease." Breckenridge, Colorado. Mar.29-Apr.4, 1993.

2. Saulnier, R., B. Bhardwaj, R. Lall, D. Leopold, L. Maxwell, and Elliott, B.E., 3T3L1 adipocytes stimulate anchorage-independent growth of a breast carcinoma. Possible role of extracellular matrix. Fifth International Congress of the Metastasis Research Society. HIH Clinical center, Bethesda M.D., USA. Sept. 28th - Oct. 1st 1994.

3. R. Saulnier, B. Chan, S. Uniyal, T. Chau, A. Patrzykat and B.E. Elliott. Fibronectin and growth factors are required for anchorage-independent growth and survival of a breast carcinoma. Oncogene meeting, 1996.

The following individuals have received pay from this effort

Dr. Ron Saulnier
Alex Patrzykat
Priti Shenoy

Fibronectin Fibrils and Growth Factors Stimulate Anchorage-Independent Growth of a Murine Mammary Carcinoma

RON SAULNIER,* BHAVNA BHARDWAJ,*,1 JENNIFER KLASSEN,* DORIS LEOPOLD,* NADER RAHIMI,* ERIC TREMBLAY,* DEANE MOSHER,† AND BRUCE ELLIOTT*,2

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Stromal cells are important regulators of mammary carcinoma growth and metastasis. We have previously shown that a 3T3-L1 adipocyte cell line secretes hepatocyte growth factor (HGF), which stimulates proliferation of a murine mammary carcinoma (SP1) in monolayer cultures (*DNA Cell Biol.* 13, 1189–1897, 1994). We now examine the role of growth factors and the extracellular matrix protein fibronectin in stimulation of anchorage-independent growth of SP1 cells. Purified transforming growth factor-β (TGF-β) stimulated significant colony growth in soft agar cultures, whereas HGF had a lesser effect. Analysis by confocal microscopy revealed that carcinoma cell colonies contained extracellular microfibrils composed of fibronectin. Partial depletion of fibronectin from 7% FBS/agar cultures reduced the number of colonies; colony growth could be recovered by adding back exogenous fibronectin. Addition of the 70-kDa N-terminal fragment of fibronectin, which inhibits fibronectin fibril formation, reduced growth of SP1 cell colonies, but an 85-kDa fragment containing the cell binding domain did not inhibit colony growth. These findings indicate that deposition of extracellular fibronectin fibrils is necessary, but not sufficient, for anchorage-independent growth of SP1 mammary carcinoma cells; growth factors are also required. SP1 cells had less fibronectin mRNA and secreted less fibronectin protein under anchorage-independent conditions than under anchorage-dependent conditions, as determined by Northern blotting and immunoprecipitation analysis. Thus, both growth factors (HGF and TGF-β) and fibronectin may be important regulators of paracrine stimulation by stromal cells of anchorage-independent growth of mammary carcinoma cells. © 1996 Academic Press, Inc.

INTRODUCTION

The mammary stroma, made up primarily of adipocytes and fibroblasts, is an important regulator of growth and development of normal and malignant epithelial cells in the breast [1–6]. Stromal cells, especially adipocytes, are in direct contact with epithelial stem cells in the terminal end buds of the mammary gland and are required for hormone responsiveness and tissue-specific differentiation of epithelial cells [reviewed in 2]. At puberty, estrogen delivers signals via stromal cells to the end bud. These signals lead to increased expression of transforming growth factor-α peptide and of epidermal growth factor receptor in epithelial cells of the terminal end bud, followed by ductal growth and side-branching [7, 8]. *In vitro*, primary mammary adipose tissue fragments [9–11] and an adipocyte cell line 3T3-L1 [11–13] can stimulate growth of normal and malignant mammary epithelial cells. However, the signaling mechanisms involved in mammary stromal–epithelial interactions are not known.

Two growth factors, keratinocyte-derived growth factor [14, 15] and hepatocyte growth factor (HGF) [16], are produced by mammary stromal cells and can stimulate ductal growth of mammary epithelial cells *in vitro*; HGF receptor is frequently up-regulated in breast cancer [17]. In addition, the ECM basement membrane protein laminin has been shown to stimulate differentiation of normal breast epithelium without growth [18] and to stimulate growth of primary breast carcinoma cells without differentiation [19]. Fibronectin (FN), though not normally expressed in basement membrane, is up-regulated in some malignancies [20, 21] and can promote a growth response to certain growth factors [22, 23]. Thus, both growth factors and extracellular matrix (ECM) are considered to be important paracrine regulators of mammary epithelial growth.

Anchorage-independent growth is a common characteristic of transformed cells. Tumor spheroids have gained interest as a result of their resemblance to tumor nodules *in vivo*. Unlike their normal cell counter-

A

B
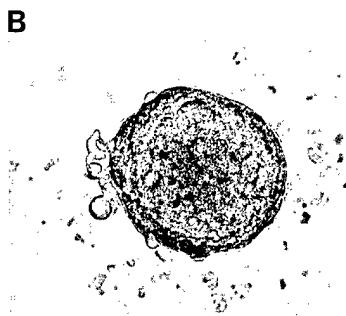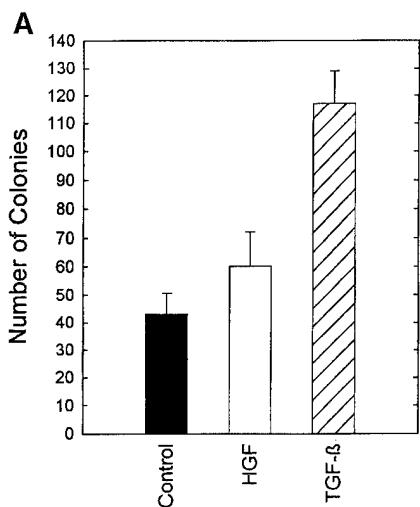
FIG. 1. Effect of purified growth factors on SP1 colony growth. (A) SP1 cells (10^3/plate) were mixed with 0.36% agar and layered onto 0.6% agar in 1% FBS/RPMI medium. Cultures were supplemented with control medium (no growth factors), HGF (20 ng/ml), or TGF-β (0.2 ng/ml). After 10–12 days, the colonies were fixed in methanol, stained with Giemsa stain, and counted manually. Results are expressed as the mean number of colonies per plate ± SD. (B) An example of SP1 cells.

part, they do not require adhesion and spreading in order to proliferate. Although a flattened cell morphology is not required for growth, tumor spheroids have ECM proteins [24, 25] and respond to growth factors [26]. The functional significance of ECM and growth factors in cell cycle progression in anchorage-independent growth is not fully understood.

We have developed a murine mammary carcinoma (SP1) model to examine mammary stromal interactions in tumor growth and invasiveness [5, 9]. We have shown that a 3T3-L1 adipocyte cell line secretes HGF, which is mitogenic for SP1 cells in monolayer cultures [27]. In addition, 3T3-L1 adipocytes secrete latent forms of transforming growth factor-β1 (TGF-β1), TGF-β2 [28], and ECM proteins [29]. These components may be important regulators of the stromal environment in breast cancer. In the study described in the

present report, we examined the effects of HGF and TGF-β on anchorage-independent growth (i.e., without cell spreading) of SP1 cells in agar cultures, a phenotype which is characteristic of malignant cells. TGF-β stimulated significant colony growth, whereas HGF had a lesser effect. Since TGF-β can stimulate expression of ECM proteins which could affect cell growth [30], we examined the possible role of ECM in SP1 colony growth. Colonies grew as tightly adhesive spheroids with prominent extracellular FN fibrils. We therefore examined the requirement for FN fibrils in the colony growth of SP1 cells and the possible source of FN in SP1 colonies. The results suggest that both growth factors (HGF and TGF-β) and ECM (FN) may be important paracrine regulators of anchorage-independent growth of SP1 cells.

MATERIALS AND METHODS

Media. Maintenance medium for SP1 tumor cell cultures was RPMI 1640 (GIBCO), with 20 mM L-glutamine (GIBCO) and 7% fetal bovine serum (FBS, Flow Laboratories). For some experiments, RPMI 1640 was supplemented with 10^{-5} M sodium selenite (Sigma), 3×10^{-5} hydrocortisone (Sigma), and 15 μg/ml transferrin (Sigma) (referred to as serum-free medium).

Cell lines. SP1 is a mammary adenocarcinoma which arose spontaneously in an 18-month-old CBA/J female retired breeder obtained from Jackson Laboratories. The histological and ultrastructural characterization of the original SP1 tumor as an infiltrating ductal carcinoma and its growth properties have been described previously [31]. A cell line was established *in vitro* and frozen down to maintain

TABLE 1

Detection of Extracellular Matrix Proteins by Indirect Immunofluorescence in SP1 Cells, 3T3-L1 Preadipocytes, and 3T3-L1 Adipocytes in Monolayer Cultures, and SP1 Colonies in Agar

ECM[a]	SP1 cell monolayer	SP1 colonies	3T3-L1 preadipocytes	3T3-L1 adipocytes
Fibronectin	+[b]	+[c]	+	+
Vitronectin	+	+	−	+
Laminin	Trace	+	+	+
Collagen IV	−	−	+	+
Tenascin	−	−	−	−

[a] SP1 cells in monolayers, SP1 colonies, 3T3-L1 preadipocytes, and 3T-3L1 adipocytes were prepared as described under Materials and Methods. ECM proteins were detected with the following primary antibodies: polyclonal rabbit anti-FN antiserum (1:100); goat anti-collagen type IV IgG (25 μg/ml); rabbit anti-laminin antiserum (1:400); rabbit anti-vitronectin antiserum (1:50), and monoclonal rat anti-mouse tenascin IgG (0.25 μg/ml). Primary antibodies were detected with appropriate FITC-conjugated secondary antibodies: goat anti-rabbit IgG (10 μg/ml), goat anti-rat IgG (25 μg/ml), and donkey anti-goat IgG (7 μg/ml). "+" indicates the presence of ECM protein; "−" indicates the absence of ECM protein.

[b] Staining was predominantly intracellular.

[c] Staining was predominantly extracellular.
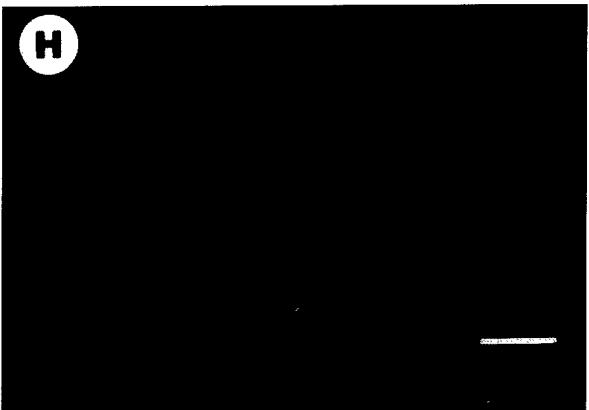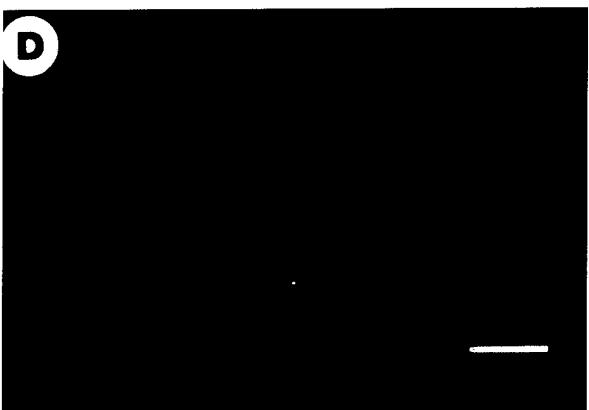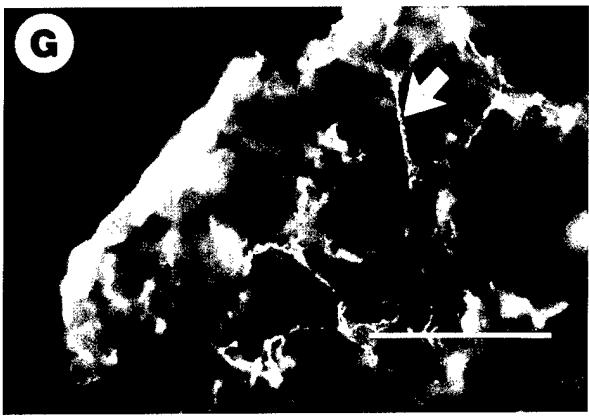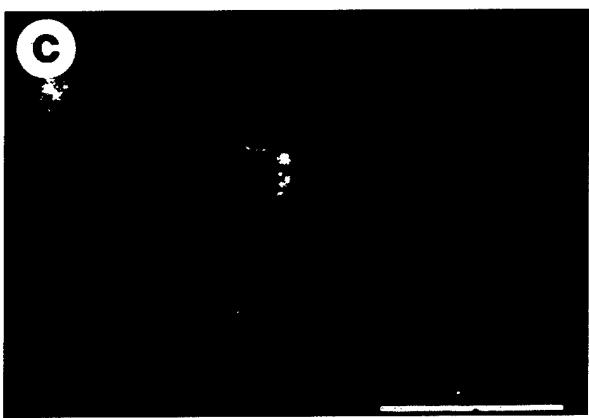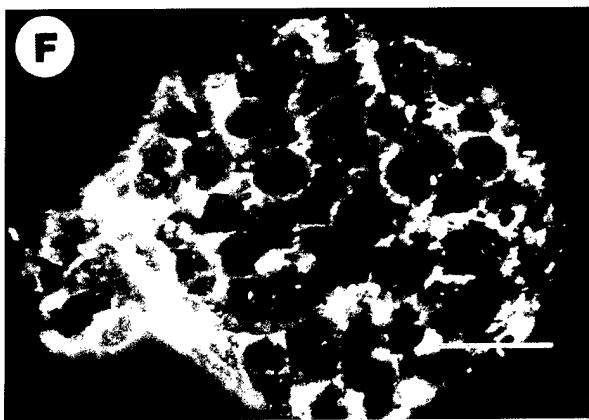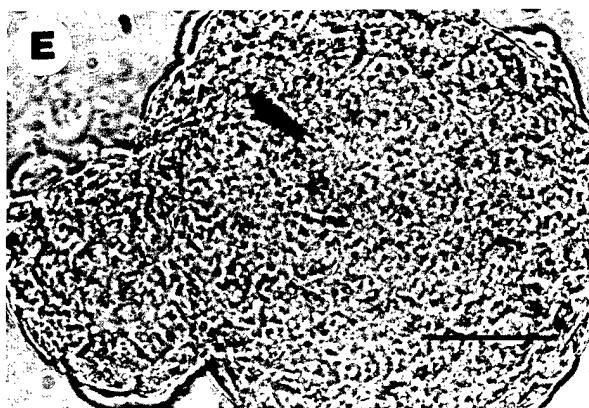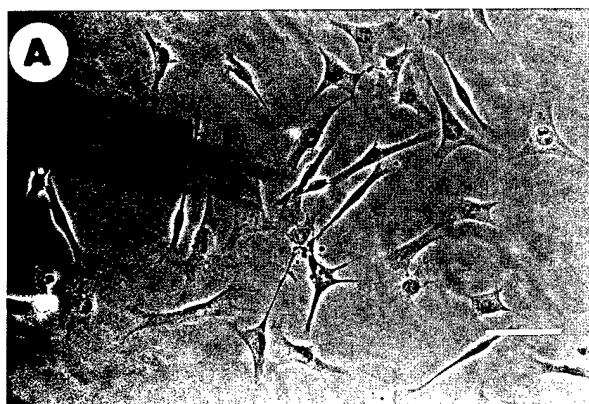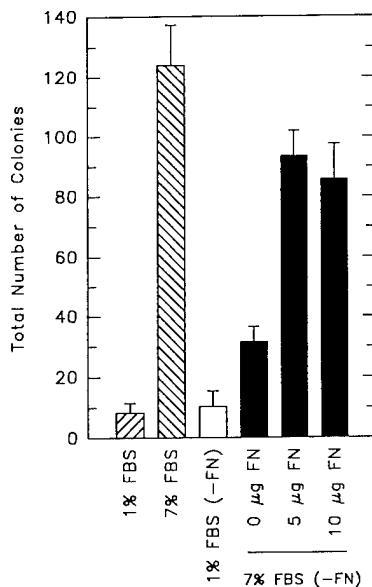
FIG. 3. Effect of partial depletion and reconstitution of FN on SP1 colony growth in 7% FBS cultures. SP1 cells (10^3 cells per plate) were cultured in RPMI supplemented with standard FBS, or FBS partially depleted of FN, at concentrations of 1 and 7%. In some cultures with 7% FBS partially depleted of FN, bovine plasma FN (5 and 10 μg/ml) was added back. Colonies were assayed after 10–12 days as described in the legend to Fig. 1. Results are expressed as the mean number of colonies of triplicate cultures $\pm$ SD.

stock. Cells were kept in culture for no more than 3 months before fresh stocks were thawed. All tumor cell lines were tested periodically for mycoplasma [31]. 3T3-L1 preadipocyte and adipocyte cell lines were cultured as described previously [27].

Assay for colony-forming cells. A liquid solution of 1.2% Bactoagar (Difco Laboratories) was mixed (1:1) with 2× RPMI 1640, supplemented to yield the final concentrations indicated of L-glutamine (20 mM) and FBS (1 or 7%), and layered onto 60 × 15-mm tissue culture plates. A 0.36% Bactoagar solution with RPMI 1640 and FBS (as above) was similarly prepared, mixed with 1 × 10^3 SP1 cells/2.5 ml, and layered on top of the 0.6% agar (1.5 ml/plate). Plates were incubated at 37°C and 5% CO_2 for 10–12 days. SP1 colonies were fixed with methanol, stained with Giemsa stain, and counted manually. In some experiments, FBS prescreened for reduced levels of FN (Sigma, Catalog No. F-2013), bovine plasma FN (GIBCO), the 70-kDa N-terminal catheptic D fragment of FN [32], and the 85-kDa tryptic cell adhesion fragment of FN [33] were used at the concentrations indicated.

Cell adhesion assay. The quantitation of cell adhesion has been described previously [23]. Briefly, a 96-well tissue culture plate (Lin-
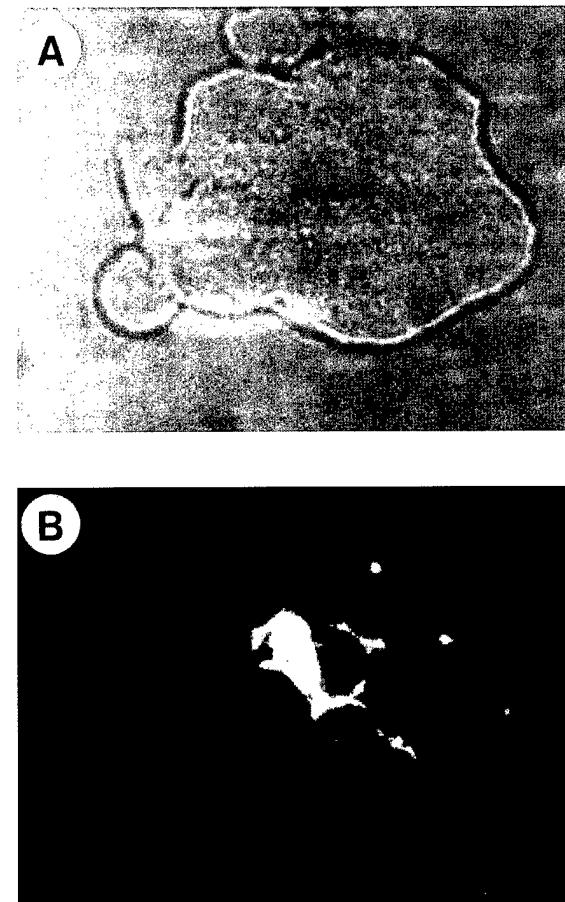
FIG. 4. Formation of fibronectin fibrils in SP1 colonies in the presence of FITC-conjugated fibronectin. SP1 colonies were grown in RPMI/agar cultures supplemented with 3% FBS partially depleted of FN and 30 μg/ml of FITC-conjugated FN. After 5 days, colonies were fixed, dried on glass slides, and viewed with a Meridian confocal microscope as described in the legend to Fig. 2. (A) Phase-contrast illumination; (B) fluorescence illumination. (Original magnification, 1000×.) Controls with unlabeled FN showed colonies with no immunofluorescence (not shown).

bro) was coated with ECM proteins for 48 h at 4°C. Remaining protein-binding sites on the plate were blocked with 0.1% BSA. Plates were washed three times in PBS and cells (10^5/well) were added in DMEM with 0.1% BSA. Cells were incubated at 37°C for 45 min. The plates were washed and the adherent cells remaining were stained with Coomassie blue and quantitated by measuring optical density at a wavelength of 270 nm in an ELISA plate reader.

FIG. 2. Immunofluorescence analysis of FN expression in SP1 colonies and monolayers. SP1 colonies or SP1 cells grown on glass coverslips were dried and fixed as described under Materials and Methods. Fixed cells were permeated with Triton-X 100 (0.03%), blocked with 1% BSA, and stained with rabbit anti-FN antiserum (1:100) and FITC-conjugated goat anti-rabbit IgG. (A) Phase-contrast photograph of cells on coverslips; (B) same field as (A) stained with rabbit anti-FN antiserum (arrow indicates region of punctate perinuclear staining); (C) SP1 cells in monolayer stained with anti-FN antiserum at higher magnification; (D) SP1 cells in monolayer stained with rat anti-tenascin IgG (0.25 μg/ml); (E) bright field of SP1 colony; (F) same field as (E) stained with anti-FN antiserum and viewed by confocal microscopy; (G) SP1 colony stained with anti-FN antiserum and viewed by confocal microscopy at higher magnification (arrow indicates extracellular FN fibril); and (H) SP1 colonies stained with anti-tenascin IgG. Bar indicates scale of 10 nm.

TABLE 2

Inhibition of Fibronectin Fibril Formation in SP1 Mono-layer Cultures with the 70-kDa N-Terminal Fragment of Fi-bronectin

Native fibronectin	70-kDa fibronectin fragment	Proportion (%) of cells with fibrils
10 μg/ml	0	87.9 ± 4.7
10 μg/ml	10 μg/ml	23.8 ± 1.5
10 μg/ml	20 μg/ml	19.9 ± 4.5

Note. SP1 cells (8×10^4) were subcultured in Nunc coverslip culture dishes with 1 ml of 0.5% FBS/RPMI culture medium containing native FN and the 70-kDa FN fragment at the concentrations indicated. After 24 h, cells were washed with serum-free RPMI and fixed in a 1% paraformaldehyde/RPMI solution (20 min). Cells were then washed and stained with rabbit anti-FN IgG and FITC-conjugated goat anti-rabbit IgG as described under Materials and Methods. Fibril formation was visualized using a Meridian confocal microscope, and the proportion of total cells with fibrils was determined manually by counting 250–300 cells per group. Results are expressed as the average of two independent experiments ± range.

Immunofluorescence staining. SP1 colonies were collected by pipetting and then dried onto glass slides. For two-dimensional cultures, SP1 cells were cultured in serum-free medium on glass coverslips coated with 0.1% (w/v) poly-L-lysine (Sigma). Dried cells were fixed with paraformaldehyde (2%) and treated with Triton X-100 (0.03%) to allow permeation of antibodies. Cells were blocked with 1% BSA and stained with one of the following ECM-specific primary antibodies: polyclonal rabbit anti-mouse FN antiserum (1:100 dilution, GIBCO), rabbit anti-mouse vitronectin antiserum (1:50 dilution) (M. Pera, Oxford University, Oxford, England), goat anti-human collagen type IV IgG (25 μg/ml) (Southern Biotechnologies), rabbit anti-mouse laminin B1/B2 chain antiserum (1:400) (UBI), and monoclonal rat anti-mouse tenascin IgG (0.25 μg/ml) (provided by Dr. P. Ekblom, Uppsala University, Uppsala, Sweden). Primary antibodies were detected by the appropriate FITC-conjugated secondary antibodies: goat anti-rabbit IgG, rabbit anti-goat IgG, and sheep anti-rat IgG (Cappel Laboratories). Fluorescence was viewed with a Leitz Aristoplan microscope equipped with epi-illumination with a mercury 100-W lamp or with a Meridian confocal microscope equipped with an argon laser. T-MAX 400 Kodak film was used.

mRNA extraction and Northern analysis. The Micro-Fast Track system (InVitrogen) was used to extract poly(A)$^+$ RNA from cultured cells or tissues. mRNA was subjected to denaturing electrophoresis on a 1.4% agarose gel and then transferred to Zeta-Probe membranes (Bio-Rad). The hybridization method used was a modification of that of Sambrook *et al.* [34]: Blots were prehybridized for 4 h at 42°C in standard hybridization solution (50% formamide, 4× Denhardt's solution, 0.25× SSC, 0.1 mg/ml sheared herring testes DNA, 1% SDS, 5 mM EDTA, 50 mM NaH$_2$PO$_4$). ^{32}P-labeled probes were prepared by a nick translation method (nick translation kit, GIBCO) and hybridized with the membrane for 24 h at 42°C in standard hybridization solution. Membranes were washed for 15 min at room temperature with each of the following: 2× SSC/0.1% SDS, 0.5× SSC/0.1% SDS, and 0.1× SSC/0.1% SDS. Autoradiograms were scanned on a laser densitometer (Molecular Dynamics). Volume analysis for specific bands was performed and normalized with values for GAPDH mRNA determined by the same methods in the same lane on the same membrane. A cDNA probe (821 bp) corresponding to two type III homology domains in the N-terminal region of the mature form of murine FN was obtained from Dr. P. Ekblom (unpublished), and

a cDNA probe (1.1 kb) for the murine laminin β1 chain [35] was obtained from Dr. K. Rubin.

Biosynthetic labeling and immunoprecipitation analysis. SP1 cells, on plastic and on agar, were biosynthetically labeled with 100 μCi/ml [^{35}S]methionine (ICN) in methionine-free RPMI medium (GIBCO) with 7% FBS dialyzed against PBS. After a 5-h incubation followed by a 2-h pulse with [^{35}S]methionine, cells were lysed in lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 μg/ml leupeptin, 2 μl/ml aprotinin, and 0.1 μl/ml 4-amidinophenylmethanesulfonyl fluoride (APMSF) (Sigma)). Conditioned medium (CM, 10 ml) from each culture was concentrated in Centricon 30 microconcentrating tubes (Amicon) in a Beckman Rotor 20.1 at 5000 rpm at 4°C. FN and laminin were sequentially immunoprecipitated from cell lysates and concentrated CM with the corresponding polyclonal rabbit anti-mouse antisera (2 μl/ml) overnight at 4°C and then with protein A–Sepharose (Pharmacia) for 3 h. Samples were boiled for 5 min and subjected to 7% SDS–PAGE under reducing conditions. The gel was treated with Amplify (Amersham) for 30 min and dried, and the autoradiogram was developed after a 2-day exposure.

RESULTS

TGF-β and HGF stimulate anchorage-independent growth of SP1 cells. Our preliminary results showed that 3T3-L1 adipocyte monolayers can stimulate anchorage-independent growth of SP1 cells in soft agar cultures (data not shown). Previously, we have shown that adipocytes secrete HGF [27] and latent forms of TGF-β1 and TGF-β2 [28], which are activated by acid treatment [36]. Since both HGF [37] and TGF-β [30, 38] have been shown to stimulate colony growth of certain tumor cells, we tested the ability of HGF and TGF-β to stimulate SP1 colony growth in soft agar cultures. The results show that TGF-β strongly stimulated colony growth, whereas HGF stimulated colony growth to a lesser extent (Fig. 1A). No additive effect was observed when both growth factors were added together to cultures (not shown). Thus, TGF-β and HGF promote SP1 colony growth.

Extracellular fibronectin fibrils are present in SP1 colonies. SP1 colonies in agar grew as tight spheroids (Fig. 1B). This morphology suggests the presence of adhesive proteins that promote close cell contact [39]. We therefore examined the presence of ECM proteins in SP1 colonies grown on adipocyte monolayers (not shown) and in 7% FBS/agar cultures (Table 1, Figs. 2F and 2G). Under both conditions, extracellular FN was detected in SP1 colonies, whereas very little cytoplasmic FN was expressed, as determined by confocal microscopy. The presence of FN fibrils was evident, particularly in 7% FBS/agar cultures (Fig. 2G). In contrast, SP1 cells in monolayer cultures revealed a perinuclear punctate pattern of cytoplasmic FN as well as some membrane-associated FN as determined by immunofluorescence staining (Figs. 2B and 2C). Punctate and diffuse expression of vitronectin and, to a lesser extent, laminin were also observed in SP1 cells in both
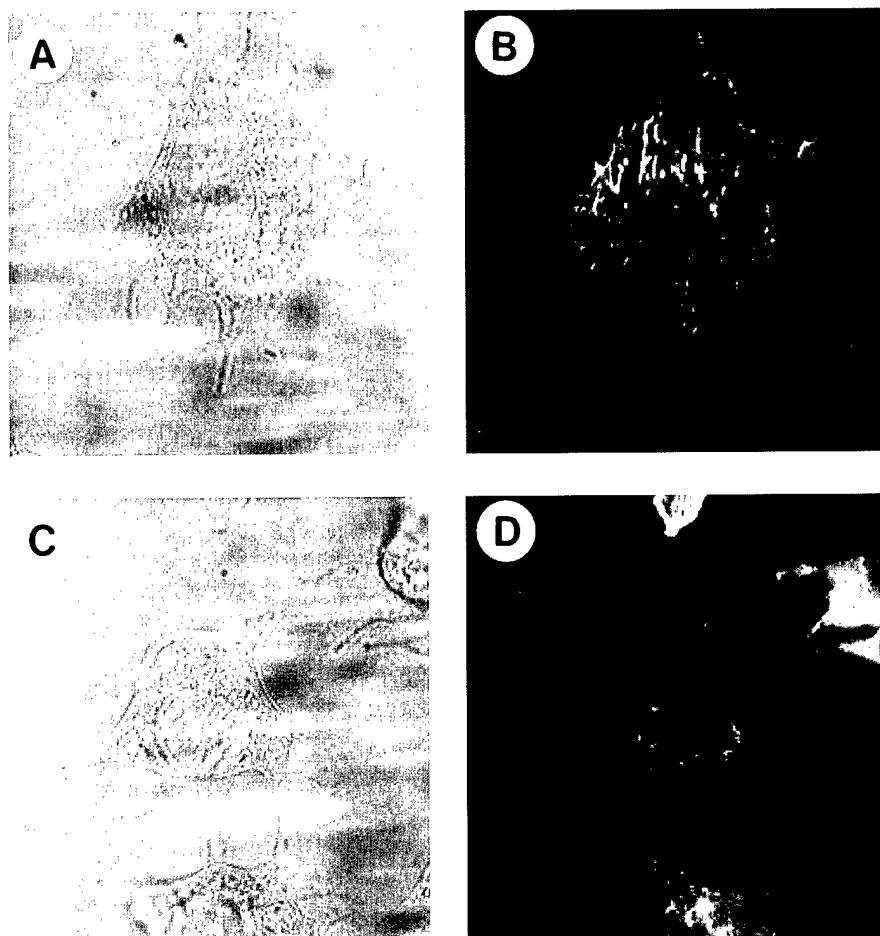
FIG. 5. Effect of 70-kDa N-terminal fragment of fibronectin on the formation of fibronectin fibrils by SP1 cells in monolayer cultures. SP1 cells (8 × 10⁴) were subcultured in Nunc coverslip culture dishes with 1 ml of 0.5% FBS/RPMI culture medium containing native FN (10 μg) without or with the 70-kDa N-terminal FN fragment (20 μg). After 24 h, cells were washed with serum-free medium, fixed in 1% paraformaldehyde/RPMI solution, and stained with rabbit anti-FN IgG and FITC-conjugated goat anti-rabbit IgG as described under Materials and Methods. Fibril formation was visualized using a Meridian confocal microscope. (A) Control cells under phase-contrast illumination; (B) control cells under fluorescence illumination; (C) cells with 70-kDa FN fragment under phase-contrast illumination; and (D) cells with 70-kDa FN fragment under fluorescence illumination. (Original magnification, 1000×.)

colonies and monolayers (Table 1). Tenascin (Figs. 2D and 2H) and collagen type IV (Table 1) were not detected in either monolayers or colonies of SP1 cells. FN is therefore a major ECM substrate in SP1 colonies.

FN fibrils are required for SP1 colony growth. We have previously shown that FN can enhance the proliferative response of SP1 cells to some growth factors (e.g., basic fibroblast growth factor) in monolayer cultures [23]. To determine the role of FN in the growth of SP1 colonies, we examined the ability of SP1 cells to form colonies in cultures with 7% FBS partially depleted of FN in the absence of any feeder cells. Under these conditions, SP1 colony growth was reduced, but could be partially recovered by adding exogenous bovine plasma FN (5–10 μg/ml) (Fig. 3). Addition of FITC-conjugated FN to the cultures resulted in a sig-

nificant incorporation of exogenous FN into the FN fibrils detected in SP1 colonies (Fig. 4). Addition of FN alone to cultures with 1% FBS only slightly stimulated cell growth (not shown); thus FN is necessary but not sufficient for optimal colony growth of SP1 cells.

To examine the role of FN fibrils in the growth of SP1 colonies, we tested the effect of the 70-kDa catheptic D N-terminal fragment of FN, which has been shown to inhibit FN fibril formation [32], on the growth of SP1 colonies in 7% FBS/agar cultures. Addition of the 70-kDa N-terminal fragment of FN to adherent SP1 cells at concentrations of 10 and 20 μg/ml resulted in a marked inhibition of FN fibril formation (Table 2, Fig. 5). Colony growth was inhibited by addition of increasing concentrations of the 70-kDa N-terminal FN fragment (Fig. 6A), but not of the 85-kDa tryptic fragment

A

70 kDa FN Fragment Conc. (μg/ml)

B

Protein Concentration (μg/ml)
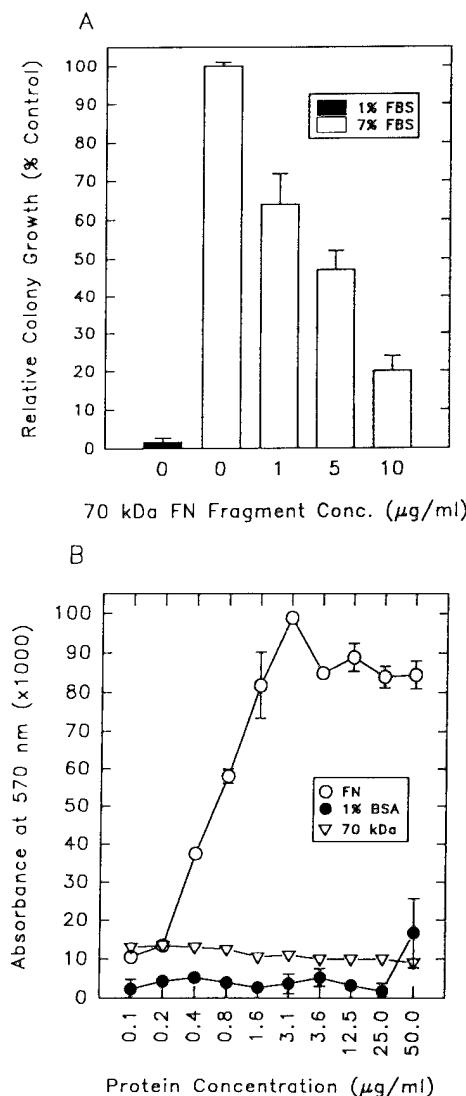
FIG. 6. (A) Effect of 70-kDa N-terminal fragment of FN on SP1 colony growth. SP1 cells were grown as colonies with 7% FBS/RPMI agar cultures in 60 × 15-mm dishes as described under Materials and Methods. The 70-kDa N-terminal fragment of FN was added at the concentrations indicated. Plates were incubated at 37°C for 10–12 days, fixed, and quantitated as described in the legend to Fig. 1. Results are expressed as the mean number of colonies per plate ± SD. (B) Comparison of adhesion of SP1 cells to native FN and to the 70-kDa N-terminal FN fragment. Linbro 96-well nontissue culture plates were coated with BSA, FN, and the 70-kDa N-terminal FN fragment at 20 μg/ml. The plates were incubated at 4°C for 48 h. The excess matrix was removed with PBS, and the wells were blocked with RPMI containing 2.5 mg/ml BSA for 2 h at 37°C. SP1 cells were added at a concentration of 3 × 10^4 cells per well in RPMI containing 2.5 mg/ml BSA. The cells were incubated at 37°C for 45 min. Unattached cells were removed with PBS and the remaining cells were fixed with 2% paraformaldehyde containing Coomassie brilliant blue for 24 h. Absorbency (270 nm) was measured in an ELISA plate reader. Results are expressed as the mean absorbency of 3 wells ± SD.

TABLE 3

Effect of the 70-kDa and 85-kDa Fibronectin Fragments on SP1 Cell Colony Growth

Fibronectin fragments	Number of colonies
3% FBS (alone)	90 ± 6.5
3% FBS + 70-kDa FN fragment (10 μg/ml)	62 ± 4.1[a]
3% FBS + 85-kDa FN fragment (10 μg/ml)	91 ± 11

Note. SP1 cells (10^3) were plated in 0.75 ml of a 1:1 mixture of agar and RPMI 1640 medium in 35 × 10-mm dishes. The final concentration of agar was 0.36%. The 70-kDa catheptic D N-terminal fragment [32] or the 85-kDa tryptic cell adhesion fragment [33] was added to the medium at 10 μg/ml before solidification. The dishes were incubated at 37°C for 10 days. The colonies were then fixed with methanol, stained with Giemsa, and counted manually. Results are expressed as the mean of triplicates ± SD.

[a] Significant reduction in number of colonies compared to control (3% FBS alone) ($P = 0.006$).

of FN containing the cell binding domain (Table 3). SP1 cells did not bind directly to substratum consisting of the 70-kDa FN fragment (Fig. 6B), nor did the 70-kDa FN fragment compete for binding of SP1 cells to native FN substratum (not shown). Thus the observed inhibition of growth with the 70-kDa N-terminal fragment of FN occurred indirectly, most likely via inhibition of FN fibril formation.

FN mRNA and protein are expressed in SP1 cells but are down-regulated under anchorage-independent conditions. The absence of cytoplasmic FN in SP1 cells in colonies compared to SP1 cells in monolayers (Fig. 2) suggests that FN synthesis is reduced under nonadherent conditions. We therefore determined the steady state levels of FN, laminin mRNA, and secreted protein in SP1 cells in monolayers (anchorage-dependent) and in colonies (anchorage-independent) in 7% FBS cultures (Fig. 7). Both FN and laminin mRNA were abundantly expressed in SP1 cells on monolayers. In contrast, expression of FN mRNA was significantly reduced in SP1 colonies, whereas the level of laminin and GAPDH mRNA remained unchanged. Only trace levels of collagen types I and IV mRNA were detected in SP1 monolayers or colonies (data not shown).

Both FN and laminin proteins were detected in immunoblots of CM from monolayers of SP1 cells (data not shown). To examine the direct effect of cell adhesion on FN and laminin production, we assessed protein synthesis and secretion of FN and laminin by SP1 cells in short-term (5 h) suspension (nonadherent) and monolayer (adherent) cultures with 7% FBS. Synthesis of [35S]methionine-labeled FN in SP1 cells was reduced 10-fold within 5 h following incubation of cells under nonadherent conditions compared to that under adherent conditions (Fig. 8A). Likewise, SP1 cells in suspension secreted 13-fold less FN than SP1 cells in mono-
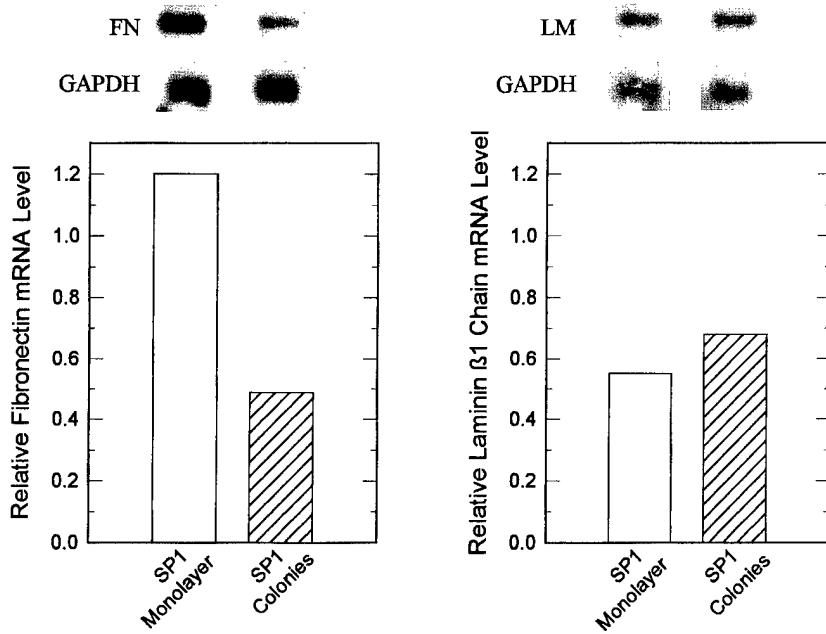
FIG. 7. Analysis of FN and laminin mRNA in SP1 cell colonies and monolayers. mRNA was prepared from SP1 cell monolayers or colonies and analyzed by Northern blotting as described under Materials and Methods. The blot was probed with cDNA for FN and laminin B1 chain. The upper bands correspond to each ECM mRNA: left, FN (8.0 kb); right, laminin B1 (9.5 kb). The lower band in all lanes is GAPDH mRNA (1.3 kb). The autoradiograms were scanned on a laser densitometer and the level of each mRNA was expressed relative to GAPDH mRNA.

layers (Fig. 8B). Synthesis and secretion of laminin were also reduced under nonadherent conditions.

DISCUSSION

Growth factors and ECM proteins are important regulators of stromal cell interactions that affect normal and malignant mammary epithelial growth. Using defined culture conditions with low serum, we have demonstrated a role of two growth factors, TGF-β and HGF, as well as the ECM protein FN in the anchorage-independent growth of SP1 cells.

Both HGF and latent TGF-β are present in 3T3-L1 adipocyte conditioned medium and have been shown previously to differentially affect SP1 cell growth in monolayer cultures [27, 28]. HGF stimulated growth [27], whereas TGF-β, activated by acid treatment, inhibited SP1 cell growth in monolayers. We now show that purified TGF-β strongly stimulated SP1 cell colony growth; HGF stimulated growth to a lesser extent. Together, these results suggest that TGF-β and HGF are important paracrine regulators of stromal interactions that affect carcinoma growth. However, interactions with other components secreted by stromal cells or SP1 tumor cells (e.g., proteinases or ECM proteins) may also modulate colony growth. Earlier reports that TGF-β, in combination with other growth factors (e.g., plate-

let-derived growth factor and basic fibroblast growth factor), stimulates anchorage-independent growth of some tumor cells [37, 38] corroborate these findings. Other effects of TGF-β on tumor microenvironment, such as increased synthesis of extracellular matrix proteins [30] and corresponding integrin receptors [30, 40], may provide a growth advantage which can overcome any direct antiproliferative effects of TGF-β [28].

Partial depletion of FN in 7% FBS-supplemented cultures reduced the number of colonies, and colony growth could be recovered by addition of exogenous FN. We also showed that exogenous FITC-conjugated FN constituted a significant component of the FN fibrils in SP1 colonies. These findings suggest that under these conditions endogenous (cellular-derived) FN is insufficient for SP1 colony growth and does not constitute a major component of FN fibrils in SP1 colonies. This contention is further supported by the observation that the FN mRNA level and FN synthesis and secretion were markedly down-regulated in SP1 cells under anchorage-independent conditions compared to those in cells under anchorage-dependent conditions. Thus, colony growth and FN fibril formation by SP1 cells appear to be dependent primarily on paracrine sources of FN (i.e., from FBS or adipocyte feeder cells).

The finding that laminin and GAPDH mRNA levels are the same in SP1 monolayers and colonies indicates

A.

B.
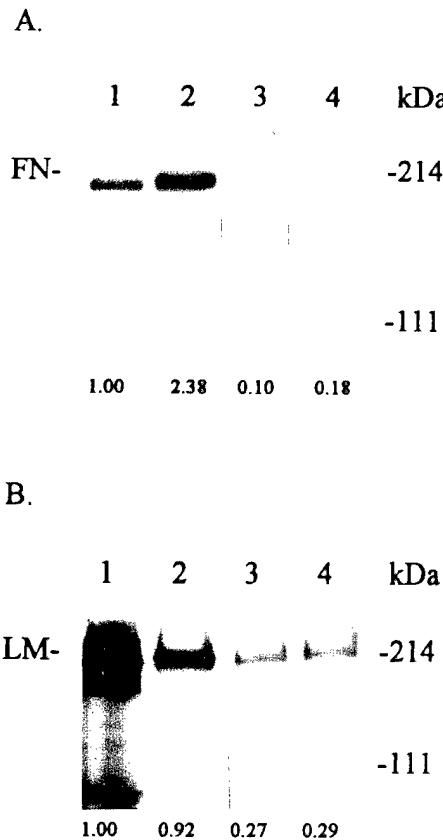
FIG. 8. Analysis of FN synthesis and secretion by SP1 cells under adherent and nonadherent conditions. SP1 cells were cultured under adhesive or nonadhesive conditions in methionine-free RPMI with 7% FBS (dialyzed against PBS) for 5 h at 37°C. During the final 2 h, [^{35}S]methionine (100 μCi/ml) was added to each culture. Cells were harvested, washed, lysed, and immunoprecipitated with rabbit anti-FN antiserum (A) or rabbit anti-laminin antiserum (B), as described under Materials and Methods. Immunoprecipitates were resuspended in sample buffer and subjected to 7% SDS–PAGE under reducing conditions. The gel was amplified, dried, and exposed for 2 days. Immunoprecipitates were from: lane 1, biosynthetically labeled adherent cells; lane 2, conditioned medium from cells in lane 1; lane 3, biosynthetically labeled nonadherent cells; lane 4, conditioned medium from cells in lane 3. The autoradiograms were scanned on a laser densitometer and the level of each protein band relative to lane 1 is indicated on the lower axes.

that the reduction in FN mRNA is related not simply to differences in growth rate. These results also suggest that both transcriptional and translational regulatory processes are involved in regulation of FN synthesis and secretion in SP1 cells. In contrast, Dalton *et al.* [41] have shown at least 50-fold reduction in FN secretion in nonadherent fibroblasts compared to that in adherent fibroblasts, while the level of mRNA and protein synthesis remained the same. In the latter system, post-translational mechanisms affecting FN assembly and secretion are likely to be involved.

Extracellular FN fibrils were present in abundance in SP1 colonies and inhibition of FN fibril formation with the 70-kDa N-terminal fragment of FN reduced colony growth. The 70-kDa FN fragment did not bind directly to SP1 cells or inhibit binding of SP1 cells to FN substratum; this result is consistent with earlier reports [32, 42] that the 70-kDa N-terminal FN fragment contains the matrix assembly site but not the cell-attachment site [43]. The 70-kDa FN fragment likely suppressed colony growth by inhibiting the formation of FN fibrils which may serve as a structural support for the cells, thereby facilitating growth. In contrast, an 85-kDa fragment of FN which contains the cell binding domain and does not inhibit fibril formation [33] did not reduce the number of SP1 colonies. These results suggest that the deposition of, and adhesion to, FN may be important events in anchorage-independent growth of SP1 cells. Extracellular FN fibrils detected *in vitro* (also called superfibronectin [44]) have greatly enhanced adhesive properties compared to those of soluble FN [45] and are the predominant form of FN in tissues [32]. FN may therefore stimulate growth of SP1 cells directly [46] or act synergistically with certain growth factors [19, 20].

The requirement for FN fibrils for SP1 colony growth suggests a partial anchorage dependence, i.e., attachment without cell spreading. Similarily, direct ligation of $\alpha5\beta1$ integrin, a major FN receptor, with anti-$\alpha5$ antibody has been shown to stimulate growth of human melanoma cells in suspension [46]. Studies with K562 cells have shown that RGD-containing peptides can stimulate cyclin A activity and anchorage-independent growth [47]. In NIH-3T3 fibroblasts, overexpression of cyclin A leads to complete loss of anchorage-dependence [48]. Thus cell adhesion is a type of "competence" signal, which may have different effects depending on cell type and ECM microenvironment; tumor cells may undergo a stepwise loss of the adhesion requirement for growth. SP1 cells appear to have lost the cell spreading requirement, but still require cell–substrate attachment and growth factor signals.

In summary, our results show that, under defined culture conditions, purified TGF-β and HGF stimulated significant colony growth. FN fibrils were also necessary but not sufficient for SP1 colony growth. Thus, paracrine regulation of SP1 colony growth may occur via both growth factors (HGF and TGF-β) and ECM (FN) components. TGF-β and HGF may affect expression/secretion of FN, thereby supporting colony growth, or these growth factors may induce signal transduction events, such as generation of cAMP [49], resulting in colony growth without any ECM requirement. Activation of latent TGF-β secreted by adipocytes could occur via proteinases from tumor or stromal cells; this possibility is currently being investigated.

Similar paracrine mechanisms may affect growth factor and ECM composition at the tumor site and thereby regulate tumor growth *in vivo*. Future work will involve identifying the growth factor receptor and ECM/integrin signaling pathways involved.

REFERENCES

1. McGrath, C. M. (1983) *Cancer Res.* **43,** 1355–1366.
2. Haslam, S. Z., Counterman, L. J., and Nummy, K. A. (1993) *Adv. Mol. Cell Biol.* **7,** 115–130.
3. Price, J. E., Polyzos, A., Zhang, R. D., and Daniels, L. M. (1990) *Cancer Res.* **50,** 717–721.
4. Vescio, R. A., Connors, K. M., Kubota, T., and Hoffman, R. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5163–5166.
5. Elliott, B. E., Maxwell, L., Arnold, M., Wei, W. Z., and Miller, F. R. (1988) *Cancer Res.* **48,** 7237–7245.
6. Miller, F. R., Medina, D., and Heppner, G. H. (1981) *Cancer Res.* **41,** 3863–3867.
7. Snedeker, S. M., Brown, C. F., and DiAugustine, R. P. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 276–280.
8. Haslam, S. Z. (1988) *J. Steroid Biochem.* **31,** 9–13.
9. Elliott, B. E., Tam, S.-P., Dexter, D., and Chen, Z. Q. (1992) *Int. J. Cancer* **51,** 416–424.
10. Beck, J. C., and Hosick, H. L. (1988) *Cell Biol. Int. Rep.* **12,** 85–97.
11. Beck, J. C., Hosick, H. L., and Watkins, B. A. (1989) *In Vitro Cell. Dev. Biol.* **25,** 409–418.
12. Levine, J. F., and Stockdale, F. E. (1984) *Exp. Cell Res.* **51,** 112–122.
13. Shappell, N. W., Lazo, R. O., and Asch, B. B. (1991) *In Vitro Cell. Dev. Biol. A* **27,** 569–577.
14. Ulich, T. R., Yi, E. S., Cardiff, R., Yin, S., Bikhazi, N., Blitz, R., Morris, C. F., and Pierce, G. F. (1994) *Am. J. Pathol.* **144,** 1–7.
15. Coleman-Kmacik, S., and Rosen, J. M. (1994) *Mol. Endocrinol.* **8,** 218–229.
16. Soriano, J. V., Pepper, M. S., Nakamura, T., Orci, L., and Montesano, R. (1995) *J. Cell Sci.* **108,** 413–430.
17. Yamashita, J., Ogawa, M., Yamashita, T., Nomura, K., Kuramoto, M., Saishoji, T., and Shin, S. (1994) *Cancer Res.* **54,** 1630–1633.
18. Streuli, C. H., Bailey, N., and Bissell, M. J. (1991) *J. Cell Biol.* **115,** 1383–1395.
19. Petersen, O. W., Ronnov-Jessen, L., Howlett, A. R., and Bissell, M. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9064–9068.
20. Elliott, B. E., Ekblom, P., Pross, H., Niemann, A., and Rubin, K. (1994) *Cell Adhes. Commun.* **1,** 319–332.
21. Oyama, F., Hirohashi, S., Shimosato, Y., Titani, K., and Sekiguchi, K. (1991) *Cancer Res.* **51,** 4876–4881.
22. Ingber, D., and Folkman, J. (1989) *J. Biol. Chem.* **109,** 317–330.
23. Elliott, B. E., Östman, A., Westermark, B., and Rubin, K. (1992) *J. Cell Physiol.* **152,** 292–301.
24. Nederman, T., Norling, B., Glimelius, B., Carlsson, J., and Brunk, U. (1984) *Cancer Res.* **44,** 3090–3097.
25. Glimelius, B., Norling, B., Nederman, T., and Carlsson, J. (1988) *APMIS* **96,** 433–444.
26. Seckl, M. J., Seufferlein, T., and Rozengurt, E. (1994) *Cancer Res.* **54,** 6143–6147.
27. Rahimi, N., Tremblay, E., Saulnier, R., Nakamura, T., Park, M., and Elliott, B. (1994) *DNA Cell Biol.* **13,** 1189–1197.
28. Rahimi, N., Tremblay, E., McAdam, L., Roberts, A., and Elliott, B. (1996) Submitted for publication.
29. Aratani, Y., and Kitagawa, Y. (1988) *J. Biol. Chem.* **31,** 16,163–16,169.
30. Chang, H.-L., Gillett, N., Figari, I., Lopez, R., Palladino, A., and Derynck, R. (1993) *Cancer Res.* **53,** 4391–4398.
31. Carlow, D. A., Kerbel, R. S., Feltis, J. T., and Elliott, B. E. (1985) *J. Natl. Cancer Inst.* **75,** 291–301.
32. McKeown-Longo, P. J., and Mosher, D. F. (1985) *J. Cell Biol.* **100,** 364–374.
33. Johansson, S. (1985) *J. Biol. Chem.* **260,** 1557–1561.
34. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Techniques in Molecular Cloning, 2nd ed., pp. 7.3–7.84, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
35. Sasaki, M., Kato, S., Kohno, K., Martin, G., and Yamada, Y. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 935–939.
36. Roberts, A. B., and Sporn, M. B. (1990) *in* Peptide Growth Factors and Their Receptors (Sporn, M. B., and Roberts, A. B., Eds.), pp. 419–472, Springer-Verlag, Berlin.
37. Galimi, F., Bagnara, G. P., Bonsi, L., Cottone, E., Follenzi, A., and Simeone, A. (1994) *J. Cell Biol.* **127,** 1743–1754.
38. Rizzino, A., Ruff, E., and Rizzino, H. (1986) *Cancer Res.* **46,** 2816–2820.
39. Albelda, S. M., and Buck, C. A. (1990) *FASEB J.* **4,** 2868–2880.
40. Rizzino, A. 1988. *Dev. Biol.* **130,** 411–422.
41. Dalton, S. L., Marcantonio, E. E., and Assoian, R. K. (1992) *J. Biol. Chem.* **267,** 8186–8191.
42. Chernousov, M. A., Fogerty, F. J., Koteliansky, V. E., and Mosher, D. F. (1991) *J. Biol. Chem.* **266,** 10,851–10,858.
43. Allen-Hoffmann, L., Crankshaw, C. L., and Mosher, D. F. (1988) *Mol. Cell. Biol.* **8,** 4234–4242.
44. Moria, A., Zhang, Z., and Ruoslahti, E. (1994) *Nature* **367,** 193–196.
45. Mosher, D. F., Sottile, J., Wu, C., and McDonald, J. A. (1992) *Curr. Opin. Cell Biol.* **4,** 810–818.
46. Mortarini, R., Gismondi, A., Santoni, A., Parmiani, G., and Anichini, A. (1992) *Cancer Res.* **52,** 4499–4506.
47. Symington, B. E. (1992) *J. Biol. Chem.* **267,** 25,744–25,747.
48. Guadagno, T. M., Ohtsubo, M., Roberts, J. M., and Assoian, R. K. (1993) *Science* **262,** 1572–1575.
49. Mochly-Rosen, D. (1995) *Science* **268,** 247–251.

THE ROLE OF INTEGRINS AND EXTRACELLULAR MATRIX IN ANCHORAGE-INDEPENDENT GROWTH OF A MAMMARY CARCINOMA CELL LINE

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Abstract - Anchorage-independent growth is a property of malignant cells. Extracellular matrix proteins are present in tumor spheroids but their function is not clearly defined. In this paper we show that a murine mammary carcinoma cell line, SP1, which expresses the fibronectin receptor $\alpha_5\beta_1$ requires fibronectin for anchorage-independent growth in soft agar. Growth factors (hepatocyte growth factor and transforming growth factor-β) also promote SP1 colony growth. In contrast, collagen types I and IV have an inhibitory effect on SP1 colony growth. A clone isolated from SP1 cells which expresses the collagen/laminin receptor $\alpha_2\beta_1$ as well as the fibronectin receptor $\alpha_5\beta_1$, demonstrates increased colony formation in the presence of fibronectin and collagen. These data suggest a role for both the $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrin receptors in the regulation of anchorage-independent growth of mammary carcinoma cells.

Key words: Fibronectin, collagen I, anchorage-independent growth, growth factors, breast cancer

INTRODUCTION

Tumor cells exhibit a number of different characteristics compared to their normal cell counterparts. The more prominent of these phenotypic changes are unlimited cell division potential, rapid proliferation, and loss of adhesive requirement for survival and growth under anchorage-independent conditions. It is well known that millions of cells can disseminate from a primary lesion into the blood stream, however, few cells actually grow as metastatic nodules in other organs (Hart and Saini, 1992;

Poste and Fidler, 1980). Folkman and Moscona (1978) have shown that normal cells need to adhere and spread in order to grow in response to growth factors; however most transformed cells have lost the cell adhesion and spreading requirement. Thus tumor spheroids cultured *in vitro* are considered to resemble *in vivo* metastatic nodules and are more representative of the tumorigenic phenotype than cells in 2-dimensional culture (Freedman and Shin, 1974). Before the characterization of integrins, several reports demonstrated the presence of extracellular matrix (ECM) proteins in tumor spheroids (De Pauw-Gillet *et al.*, 1988; Glimelius *et al.*, 1988); however, the role of integrins and interactions with growth factors in growth regulation under anchorage-independent conditions has not been thoroughly investigated.

Abbreviations: Coll I: collagen type I; **ECM:** extracellular matrix; **FBS:** fetal bovine serum; **FN:** fibronectin; **HGF:** hepatocyte growth factor; **LM:** laminin; **TGF-β:** transforming growth factor-β; **VN:** vitronectin

THE ROLE OF EXTRACELLULAR MATRIX AND GROWTH

FACTORS IN ANCHORAGE-INDEPENDENT GROWTH OF A

MOUSE MAMMARY CARCINOMA CELL LINE

By

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ABSTRACT

Anchorage-independent growth is a property of malignant cells, however little is known about how tumor cells are able to survive and grow anchorage-independently. We have previously shown that tumor spheroids growing in agar contain extracellular matrix (ECM) proteins, especially fibronectin (FN) (Saulnier *et al.*, ECR. 222:360-369, 1996). In this thesis we investigate the role of ECM proteins and growth factors in anchorage-independent survival and growth of a murine mammary carcinoma-derived cell line, designated SP1. We demonstrate that SP1 cells adhere well to FN and vitronectin (VN) and express a number of integrins, including $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_6\beta_1$, $\alpha_3\beta_1$ and $\alpha_2\beta_1$, the latter being upregulated under anchorage-independent conditions. Transforming growth factor-β (TGF-β), but not hepatocyte growth factor (HGF), could increase the adhesive potential of SP1 cells to collagen type I (Coll I) when treated under anchorage-independent conditions. We also demonstrated by partially removing FN from 7% FBS (optimal conditions) or adding FN to 1% FBS (limiting conditions) that FN is required for SP1 cell colony growth. A 70 kDa amino-terminal fragment of FN, which inhibits fibril formation, inhibited colony growth showing that FN fibril formation is also required.

Cl-12-H, an SP1 cell clone with efficient colony forming ability, was able to adhere more strongly to Coll I and expressed higher levels of $\alpha_2\beta_1$ than SP1 cells or the clone Cl-24-L, derived from SP1 cells. FN and Coll I were both able to promote colony growth of Cl-12-H cells, but had no effect on Cl-24-L cells.

We also demonstrated that soluble FN as well as HGF, and TGF-β could all promote survival of SP1 cells under anchorage-independent conditions. FN and HGF together, acted in a additive fashion on cell survival. Using either wortmannin or Cl-12-H

transfected with a dominant negative mutant p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase), we demonstrated that PI 3-kinase is only slightly involved in FN or HGF mediated survival. In contrast, the additive survival effect mediated by FN+HGF together was very dependent on PI 3-kinase.

We initiated some studies investigating candidate signaling molecules involved in anchorage-independent survival and growth. HGF receptor phosphorylation was reduced under anchorage-independent conditions but showed increased tyrosine phosphorylation in response to exogenous HGF. In contrast, focal adhesion kinase (FAK) was not phosphorylated in response to FN or HGF under anchorage-independent conditions. PI 3-kinase showed reduced phosphorylation under anchorage-independent conditions and this tyrosine phosphorylation was not increased in response to FN or HGF. In contrast, PI 3-kinase activity was increased in a stepwise manner in the presence of FN, HGF and FN + HGF. Together these results demonstrate that ECM-integrin interactions and growth factors are involved in anchorage-independent survival and growth of SP1 cells.

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LIST OF ABBREVIATIONS

BMA5:	Blocking mouse alpha 5 antibody
BSA:	Bovine serum albumin
CHO:	Chinese hamster ovary
Cl-12-H:	Clone number 12 with high colony forming ability
Cl-24-L:	Clone number 24 with low colony forming ability
Coll I/IV:	Collagen type I/IV
cpm:	Counts per minute
EDTA:	Ethylenediaminetetra-acetic acid
ECL:	Enzyme-linked chemiluminescence
ECM:	Extracellular matrix
EGF:	Epidermal growth factor
EHS:	Engelbreth Holmes Swarm
ELISA:	Enzyme-linked immunosorbant assay
FAK:	Focal adhesion kinase
FBS:	Fetal bovine serum
FGF:	Fibroblast growth factor
FITC:	Fluorescein isothiocyanate
FN:	Fibronectin
HGF:	Hepatocyte growth factor
IGF:	Insulin-like growth factor
K:	Lysine
LM:	Laminin
MHC:	Major histocompatibility complex
MDCK:	Madine Darby Canine Kidney
MMP:	Matrix metalloproteinase
NGF:	Nerve growth factor
NP40:	Nonidet P40
NRK:	Normal rat kidney
Δp85:	p85 subunit of PI 3-kinase with a mutation in the p110 binding domain
P:	Proline
PAI:	Plasminogen activator inhibitor
PBS:	Phosphate buffered saline
PDGF:	Platelet-derived growth factor
PI 3-kinase:	Phosphatidylinositol 3-kinase
PLC:	Phospholipase C
PLL:	Poly-L-Lysine
PMSF:	Phenylmethyl sulfonyl fluoride
PY:	Phosphotyrosine
RGD:	Arginine-Glycine -Aspartic acid
RIPA:	Radioimmunoprecipitation assay
rpm:	Revolutions per minute
S:	Serine
SD:	Standard deviation

SH2:	Src homology domain 2
SH3:	Src homology domain 3
TGF-β:	Transforming growth factor-beta
TLC:	Thin layer chromatography
V:	Valine
VN:	Vitronectin

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I dedicate this thesis on breast cancer research to my friend Claudette Belliveau who passed away on March 8th, 1989 at the age of 42 with breast cancer which had metastasized to her lungs. Her pain and suffering are not soon forgotten!

INTRODUCTION

Breast cancer is the most common cancer among North American women. It is the number two cause of death (after lung cancer) of women ages 35-54 and affects approximately 1 in 9 women in their lifetime; of these one third will die of metastases. Unlike lung and skin cancer, the causes of breast cancer are not well understood. Genetic factors contribute to approximately 5-10% of breast cancer cases (Cannon-Albright and Skolnick, 1996; Teixeira *et al.*, 1996), however, the etiology of the majority of cases are not known. In addition of 50% of the women who die of metastases showed no signs of metastases (node-negative) at the time of diagnosis.

Early detection is the preferred method of preventing metastatic spread, but not all tumors are caught at an early stage. Breast tumors metastasize frequently to the lungs, liver and bone. Once at distant sites, the tumor cells may grow as spherical nodules or more diffusely as in bone marrow and destroy the existing tissue. It is because of metastases that most breast cancer patients die. The most important issue in the management of primary breast cancer today is identification of individuals who harbor micrometastatic disease. Conventionally, the presence of tumor cells in axillary lymph nodes has been the best predictor of systemic micrometastases (Seidman, 1996). Conventional treatment modalities such as surgery, chemotherapy and radiation, whether used alone or in combination are not always effective at destroying all of the metastatic lesions. There has been little change in the mortality rate in the last 20 years despite many different treatment modalities (Parker *et al.*, 1997). A better understanding of metastatic cells and the ability of transformed cells

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to grow in multicellular spheroids is required before effective treatments for breast cancer are developed.

1.1 - EXTRACELLULAR MATRIX

The extracellular matrix (ECM) is composed primarily of collagens, glycoproteins and proteoglycans. ECM is not just the glue between cells as previously thought, but provides the scaffolding, support and strength to tissues and organs. In addition, almost all cellular events occuring during embryonic development and organogenesis, tissue remodeling, wound healing or tumor growth and metastasis are in some way influenced by individual components of the ECM. Various ECM proteins have also been implicated in cellular processes including cell adhesion, survival, growth, differentiation, migration and invasion (von der Mark *et al.*, 1992; Lester and McCarthy, 1992).

Structurally, ECM proteins are composed of multiple domains. Many of these domains show identity with other domains in the same protein or with domains in other proteins. The fibronectin type I, II, and III domains, epidermal growth factor-like domain, Ca^{2+} -binding and cysteine-rich domains are examples of common domains found in many ECM proteins (reviewed in Engel *et al.*, 1994). In addition to interactions with cells, ECM proteins commonly assemble into mutlimers in both homotypic and heterotypic fashion. Thus, they have binding sites for other extracellular matrix proteins (see Figure 1 for example) and growth factors (Taipale and Keski-Oja, 1997). Many of these matrix-matrix interactions form the basis for the complex ordered structure of ECM as found in basement membranes and other tissues.

This thesis is concerned primarily with how the ECM can affect cell behavior and

growth factor response. There are three mechanisms by which ECM can regulate cell behavior; (1) composition of ECM, (2) regulation of cell surface receptors that mediate adhesion to ECM, and (3) synergistic interactions between growth factors and ECM (Adams and Watt, 1990). These mechanisms are not mutually exclusive and all three can occur simultaneously.

In the resting mammary gland, modifications of the ECM influence end bud development, epithelial cell proliferation and ductal branching. During pregnancy and lactation, an intact basement membrane is required for the emergence of a differentiated phenotype. More specifically, the E3 fragment of laminin can induce β-casein expression in mammary epithelial cell (Streuli *et al.*, 1995). Finally after weaning, degradation of the ECM by up-regulation of the metalloproteinases stomelysin and collagenase, and a decrease in the tissue inhibitor of metalloproteinase (TIMP) triggers the massive programmed cell death which occurs during involution, an event which brings the gland back to its original resting state (Howlett and Bissell, 1993; Talhouk *et al.*, 1992). These results show that ECM influence mammary cell function *in vivo*.

A) Changes in ECM during disease and neoplasia

The production of ECM proteins is dynamic and influenced by a large number of factors including cytokines, growth factors and the ECM itself. Therefore during altered states such as wound healing and disease, including neoplasia, there is often deregulated synthesis and secretion of ECM proteins which will in turn affect the function of cells (Raghow, 1994; Alitalo and Vaheri, 1982). Some diseases such as liver fibrosis,

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glomerulosclerosis and interstitial fibrosis have an increase in the secretion of ECM proteins (Bruijn and Heer, 1995) while other diseases such as malignant transformation are usually associated with a reduction in synthesis and secretion of ECM proteins (Alitalo and Vaheri, 1982).

In the normal breast, the parenchyma of a functional mammary gland contains three epithelial compartments; luminal epithelium, alveolar epithelium and myoepithelium. The luminal epithelium lines milk-collecting ducts and the cells are separated from basement membrane and from stromal ECM by a layer of myoepithelium. The alveolar epithelium, which is responsible for milk production, interacts directly with myoepithelial processes that form a sparse network around alveoli, and also with laminin rich basement membrane that separates both of these cell types from the stroma (Pullan *et al.*, 1996). The surrounding stroma is composed primarily of adipose cells, fibroblasts and connective tissue containing various ECM proteins of Coll I and FN.

In the malignant breast, the cells grow continuously, are not polarized, and often fail to deposit the basement membrane proteins LM and Coll IV. In addition, there is typically an increase in Coll I, III and V, FN, thrombospondin, tenascin and VN (Lochter and Bissell, 1995). Christensen *et al.*, (1988) have shown an increase in FN staining in the stroma of some invasive breast carcinomas. They observed that 27 of 31 women who died without evidence of metastatic spread had FN-positive invasive breast carcinoma as compared to 15 of 46 women which had metastatic disease at the time of diagnosis. These results suggest that FN distribution in breast carcinomas may be used as a prognostic factor (Christensen *et al.*, 1988)

B) The structure and function of fibronectin

One of the most studied ECM proteins with respect to its distribution and function during both normal and pathological processes is fibronectin. Up to 20 different FN polypeptides can be generated by differential splicing of the mRNA transcribed from a single gene (Schwarzbauer *et al.*, 1987). Fibronectin is an important multifunctional extracellular matrix glycoprotein (Mr 400,000) composed of 2 similar chains and linked together at the carboxyl terminus by disulfide bonds (Ruoslahti, 1988). FN can be present in soluble form in plasma and other body fluids or in insoluble form in the extracellular matrix (cellular) (Ruoslahti, 1984). Fibronectin is present in some basement membranes but has not been isolated in the EHS tumor extracellular matrix (Matrigel) or the glomerular basement membrane (Laurie *et al.*, 1982; Kleinman *et al.*, 1986). The FN molecule possesses several binding domains including two fibrin and heparin binding domains, a collagen and gelatin binding domain, and two cell binding domains (Figure 1A).

At the amino acid level, FN is composed of 3 different types of internal repeats designated I, II and III which contain approximately 40, 60 and 90 a.a. residues respectively (Patel *et al.*, 1987). One of the type three repeats contains the cell binding region. Peptides cleavage of FN in this region demonstrated that the tri-peptide RGD could support adhesion of cells (Ruoslahti and Pierschbacher, 1987). Although the peptide RGD is sufficient to mediate adhesion, the flanking regions are also required for maximal adhesion. A synergy site in the 9th FN type III domain which facilitates RGD-dependent adhesion is also required for maximal adhesion to FN (Aota *et al.*, 1994). The other cell

binding site is found in an alternatively spliced region of the FN molecule and is called the IIICS domain (Guan and Hynes, 1990; Schwarzbauer *et al.*, 1987).

Fibronectin is involved in a number of normal biological functions such as cell migration (Lacovara *et al.*, 1984), adhesion, cell invasion (Ruoslahti, 1984), morphogenesis and developmental regulation (Ruoslahti,1988). Fibronectin expression, as well as that of other matrix proteins are spatially and temporally regulated during development and altered during processes such as wound healing.

A number of factors regulate FN synthesis and secretion. Fibronectin synthesis can be increased by dexamethasone (a synthetic glucocorticoid) which stabilizes the mRNA. Forskolin (an activator of adenylate cyclase) and TGF-β increase FN expression via increased gene transcription (Dean *et al.*, 1988).

The expression of FN is greatly inhibited by neoplastic transformation in most cases examined. For example, the loss of FN from the cell surface after transformation results in decreased cellular adhesion which has been correlated with increased metastasis of some tumor cell types (Roos, 1987; Akiyama *et al.*, 1995).

Fibronectin expression in the breast is located in the mesenchymal compartment where it can be detected during embryonic development and in the postnatal breast (Lochter and Bissell, 1995). In mammary tumors, FN accumulates in the stroma as a result of desmoplasia. FN synthesis in tumors is often characterized by expression of the alternatively spliced ED A and ED B regions (Mardon *et al.*, 1993; Oyama *et al.*, 1993).

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C) Fibronectin matrix assembly

To become an insoluble matrix molecule, FN has to be recaptured onto the surface of a cell capable of promoting matrix assembly. The exact mechanism of matrix assembly is not fully understood but it is thought to involve binding of FN to an integrin and to a receptor named "matrix assembly receptor"(Ruoslahti, 1994). Transfection of CHO cells with the $\alpha_5\beta_1$ integrin restored their ability to assemble FN fibrils while transfection with other integrins did not support fibril formation. Fibronectin matrix assembly is not completely dependent on $\alpha_5\beta_1$ integrin since some cells are still able to assemble a FN matrix in the absence of $\alpha_5\beta_1$ (Wu *et al.*, 1993). Wu *et al.*, (1995) have shown that $\alpha_3\beta_1$ can also function in matrix assembly.

In the extracellular matrix of embryonic and adult tissues, FN is usually present as fibrils. The amino terminal domain of FN, comprising the first five type I modules is an absolute requirement for the incorporation of FN into fibrils. Other sites including the RGD site and the first type III module are also important but not essential for matrix assembly. Only dimeric FN is incorporated into fibrils, hence the carboxyl-terminal cysteine residues involved in subunit crosslinking are essential for matrix assembly (Schwarzbauer, 1991).

Fibril formation in the Xenopus blastocoel was inhibited by RGD peptides, an amino-terminal fragment of FN and cytochalasin B. Unbranched FN fibrils grow only at one end at a rate of about 4.7 μm/min as determined by double-labelling experiments. Most fibrils grow for only a short time, and the increase in total fibril length per cell is driven by the repeated initiation of new fibrils. Assembly of FN into fibrils precedes

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crosslinking of FN into multimers in this system (Winklbauer and Stoltz, 1995).

The 70 kDa amino-terminus fragment of FN (see Figure 1) which contains the matrix assembly region can inhibit FN fibril formation by causing chain termination. The 70 kDa fragment can bind to the cells (McKeown-Longo and Mosher, 1985) but the receptors have not yet been clearly identified, however candidate molecules of 66 and 48 kDa have been isolated (Moon *et al.*, 1994). It is thought that unfolding of the type III-1 module on the cell surface may control matrix assembly site recognition and represent an important step in the initiation of cell dependent FN polymerization (Hocking *et al.*, 1994).

D) The structure and function of collagens

Collagens comprise a large heterogenous class of molecules. Some collagens demonstrate structural properties classically attributed to ColI but others have additional properties. Vertebrates contain at least 19 different types of ColI. Collagens are distinct from other proteins in that the molecule is composed of three polypeptide chains (α chains) which form a unique triple-helical structure. The smallest amino acid, glycine is present at every third residue along each chain for the three chains to be able to wind into a triple helix. Each of the three chains therefore has the repeating structure Gly-Xaa-Yaa, in which Xaa and Yaa can be any amino acid but are usually proline and hydroxproline (Kadler *et al.*, 1996).

The collagens are classified into several groups (1) the fibrillar collagens; types, I, II, III, V, and XI, (2) the fibrillar-associated collagens, types IX and XII, (3) the

network forming collagens, type IV, (4) beaded filaments, type VI, (5) the short chain collagens, types VIII, and X, and (6) the long chain collagens, type VII. The shape and most of the structural properties of a native CoII molecule are determined by its triple-helical domain(s). In the classical fibril-forming collagens a single triple-helical domain comprises more than 95% of the molecule. Other collagens, however, have multiple triple-helical domains.

Collagen type I $[\alpha1(I)]_2\alpha2(I)$, is found throughout the body execpt in cartilaginous tissues. It is a major constituent of the extracellular matrix of bone and skin. Its synthesis is restricted to predominantly two cell types; osteoclasts and fibroblasts. Changes in synthesis of CoII I occur during embryonic development and in response to injury and in many pathological conditions such as liver and lung fibrosis and scleroderma (Kadler *et al.*, 1996; Galéra *et al.*, 1994) and show an increase in the stroma of breast carcinomas (Lochter and Bissell, 1995).

Collagen type IV is the predominant structural component of basement membranes and is ubiquitous to these structures. Similar to fibrillar collagens, it is also composed of three chains in an α-helix $[\alpha1(IV)]_2\,\alpha2(IV)$. An interesting feature of collagen IV is that four other $\alpha(IV)$ chain isoforms have been discovered and are often expressed during pathogenesis. In contrast to fibrillar collagens, the newly synthesized CoII IV molecules do not undergo extracellular processing before being incorporated into the matrix. Another distinct feature is that the molecules are linked together to form a flexible network-like structure onto which other basement membrane proteins are attached (Hudson *et al.*, 1993).

1.2 - INTEGRIN RECEPTORS

Cell adhesion and spreading are required by non-transformed cells to grow and proliferate. There is a direct relationship between the degree of spreading and proliferation rate in cultured fibroblasts (Folkman and Moscona, 1978). Cell adhesion can be mediated by a number of different classes of molecules including (1) Ig superfamily, (2) cadherins (3) integrins, and (4) selectins.

A) Structure, classification and general function of integrins

Interactions between cells and the extracellular matrix are mediated primarily by cell surface receptors which belong to a superfamily of adhesion receptors called integrins.

Integrins are transmembrane glycoprotein heterodimers composed of an α and β subunit noncovalently bound together which mediate cell-cell and cell-matrix interactions. Associations of the α and β subunits are not random, although α_v associates with multiple β subunits, most α subunits associate with only one β subunit (Figure 2 and Table 1) (Hynes, 1987, Albelda and Buck, 1990). Both the α and β subunits possess large extracellular domains, a single transmembrane domain and short cytoplasmic domains (25-50 amino acids), with the exception of the β_4 subunit which has a 118 kDa cytoplasmic domain (Hogervorst et al., 1990; Suzuki and Naitoh, 1990). All α subunits contain several cation binding domains in the extracellular region. The β subunit extracellular domains contain a repetitive amino acid sequence which contains a high number of cysteine residues (Figure 1B)

Integrins were initially classified according to their β subunits and formed three

major groups: β_1, β_2, and β_3. The integrins sharing the β_1 subunit are the largest group

having nine different α subunits and bind primarily fibronectin, collagens and laminin

(Table 1). The leucocyte adhesion molecules, $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_X\beta_2$ are only found on

lymphoid and myeloid cells and share a common β_2 subunit (Larson and Springer, 1990).

The observations that the α_v subunit could associate with multiple β subunits has forced

a new classification for integrins where the β_3 family is replaced with the α_v family (Figure

2).

Integrins play a key role in a number of biological processes where such as

embryogenesis, wound healing, cell differentiation, lymphocyte migration, survival, cell-

cell interactions, cell migration and gene transcription (reviewed in Ruoslahti, 1991;

Giancotti and Mainiero, 1994). Integrins are also involved in a number of pathological

processes such as bacterial cell invasion (Isberg and Leong, 1990) and tumor cell migration

and invasion (Dedhar, 1990; Dedhar and Saulnier, 1990; Ruoslahti and Pierschbacher,

1987).

Integrins are crucial for embryonic development. A number of integrin null mice

embryos have been established recently. The β_1 null mutant is the most lethal, showing

peri-implantation lethality at day 5 (Stephens *et al.*, 1995). An α_5 null mutant showed

failure to form a notochord and somites in the posterior region distal to somite 10. Null

mutants of the α_3, α_v and α_8 showed neonatal lethality while no phenotypic changes were

observed during development or in adulthood in α_1 null mice (Fässler *et al.*, 1996).

Integrin function is regulated by a large number of different mechanisms which

ultimately involve changes in the conformational state of the protein. The major difference

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in integrin regulation is its activation state. The $\beta 1$ and $\beta 2$ integrins on hematopoietic cells and platelets are not constitutively active i.e. unable to bind ligand until they are activated by other receptors. In platelets, activation by thrombin will activate the $\alpha_{IIb}\beta_3$ and enable it to bind a number of different ligands. In contrast, on epithelial cells, integrin receptors are constitutively active. This is not a function of the integrin itself but a result of factors in the cytoplasm since transfected $\alpha_L\beta_2$ into epithelial cells will be constitutively active (Larson *et al.*, 1990). The $\alpha_5\beta_1$ receptor has multiple activation states in K562 cells where it can bind with low affinity ($K_d > 1 \mu M$) or be activated to bind with high affinity (K_d 54 nM) (Faull *et al.*, 1993). The c-Ras protein has been postulated as being an important regulator of integrin activation in myeloid cells (Zhang *et al.*, 1996).

Another important mechanism for regulating integrin activity is the presence of divalent cations. The α subunit contains 4-7 cation-binding sequences, similar to the EF-hands motif found in calcium-binding proteins such as calmodulin. The divalent cations Ca^{2+}, Mg^{2+}, and Mn^{2+} have been shown to be essential for integrin function and differentially regulate adhesion. Ca^{2+} tends to be a negative regulator while Mg^{2+} and Mn^{2+} increase integrin-mediated adhesion. It is thought that both Mg^{2+} and Mn^{2+} support ligand binding because they shift the conformational equilibrium between active and inactive states in favor of the active state while Ca^{2+} causes changes which favor the inactive state (Mould, 1996).

Phospholipid composition may be another mechanism by which integrin activity is regulated. The $\alpha_v\beta_3$ integrin inserted into liposomes bound to different ligands depending on the composition of the phospholipids in the plasma membrane. The $\alpha_v\beta_3$ integrin only

bound to VN in liposomes composed of phosphatidylcholine but was able to bind other substrates when the liposomes also contained phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and cholesterol (Conforti et $al.$, 1990).

A number of factors affect surface expression of integrins including a number of cytokines and growth factors such as TGF-β (Heino et $al.$, 1989; Roberts et $al.$, 1992). Adhesion also increases transcription of integrin genes as well as their surface expression (Chen et $al.$ 1992; Delcommenne and Streuli, 1995). Cell surface gangliosides may also be involved in integrin regulation. The GM3 ganglioside was shown to increase $\alpha_5\beta_1$-mediated adhesion to FN in the mouse mammary carcinoma cell line FUA169 (Zheng et $al.$, 1993). Proteolytic cleavage (Kishimoto et $al.$, 1989) or variations in glycosylation of the α or β subunits (Chammas et $al.$, 1993; Kawano et $al.$, 1993; Lampe et $al.$, 1993) can also affect the function of integrins. In conclusion integrins form a large family of cell surface receptors whose ability to bind to its ligand is regulated by several mechanisms.

Figure 1. The structure of fibronectin and integrin subunits

Panel A

The modular structure of fibronectin, showing the position of FN1, FN2 and FN3 modules. The position of the EDA, EDB and IIICS alternatively spliced regions are also shown. The RGD sequence is located in the 10th FN3 module. The location of the 70 and 85 KDa fragments are also indicated (From Potts and Campbell, 1994).

Panel B

General structure of the integrin α and β subunits. The α subunits are generally larger than the β subunits The location of divalent cation binding sites and cysteine rich domains are indicated as well as the domain and protease cleavage site (found only in some integrins).

Figure 1

A

70 kDa   85 kDa

Crosslinking site   Fibrin   Matrix assembly   EDB   EDA   Cell   IIICS   COOH

RGD

NH₂   COOH

Matrix assembly   Collagen   Cell   Heparin   Fibrin
Staphylococcus   Gelatin   Chondroitin
aureus   sulphate
Heparin

☐ Type 1 module   ◯ Type 2 module   ⬭ Type 3 module
   Fn1   Fn2   Fn3
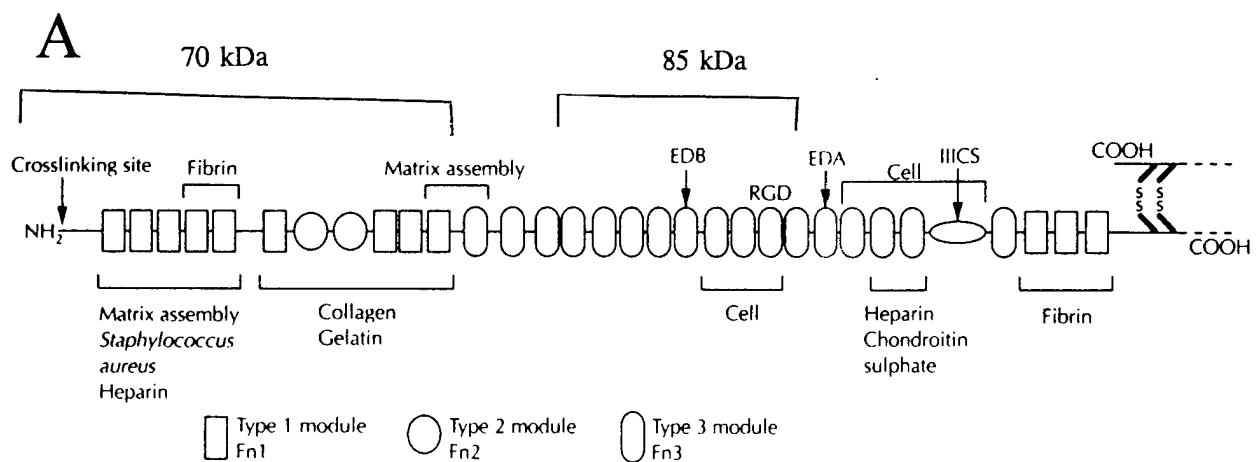
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B

                               Mg²⁺/Ca²⁺
          Repeated domains   binding domains
             I-VII   (IV-VII or V-VII)   ┌S — S┐

α-Subunit | I | II | | III | IV | V | VI | VII | | Large chain | Small chain |

                 Inserted or   Protease cleavage
                 I domain   (only some integrins)
             (only some integrins)

Signal   Trans-   Cytoplasmic
sequence   membrane   domain
                                   domain

β-Subunit | 1 | 2 | 3 | 4 |

                                Cysteine-rich
                                 domains
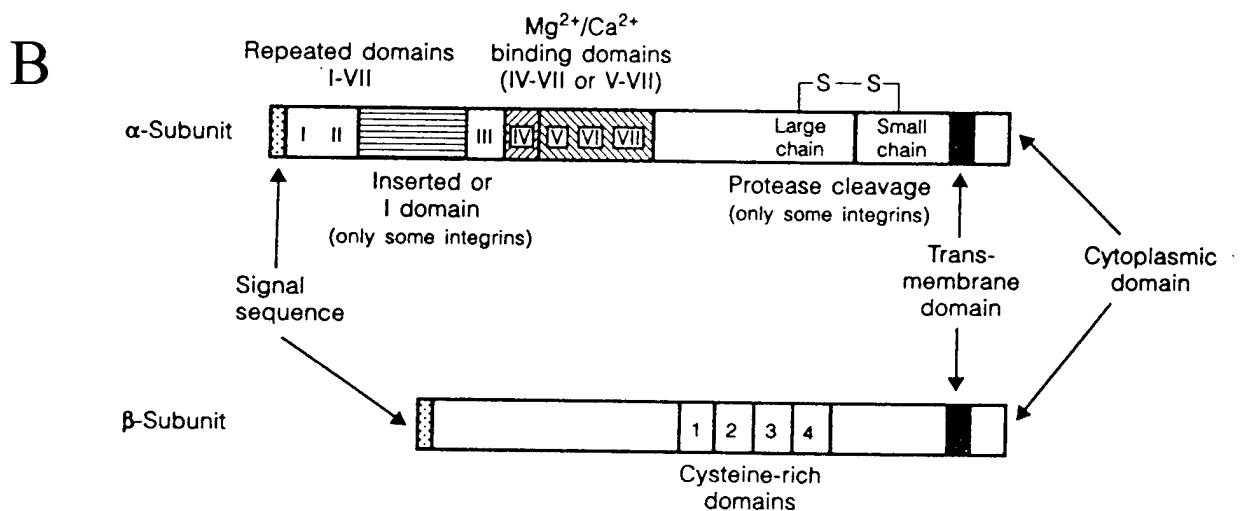
Figure 2. Integrin subunit association

The association of integrin α and β subunits (solid lines) is not random. There are approximately 21 integrin heterodimers which have been characterized to date. Note that the β_1 and β_2 subunits bind to several α subunits and that most α subunits associate with only one β subunit, with the exception of the α_v subunit which binds to several β subunits.

Figure 2

Integrin subunit association
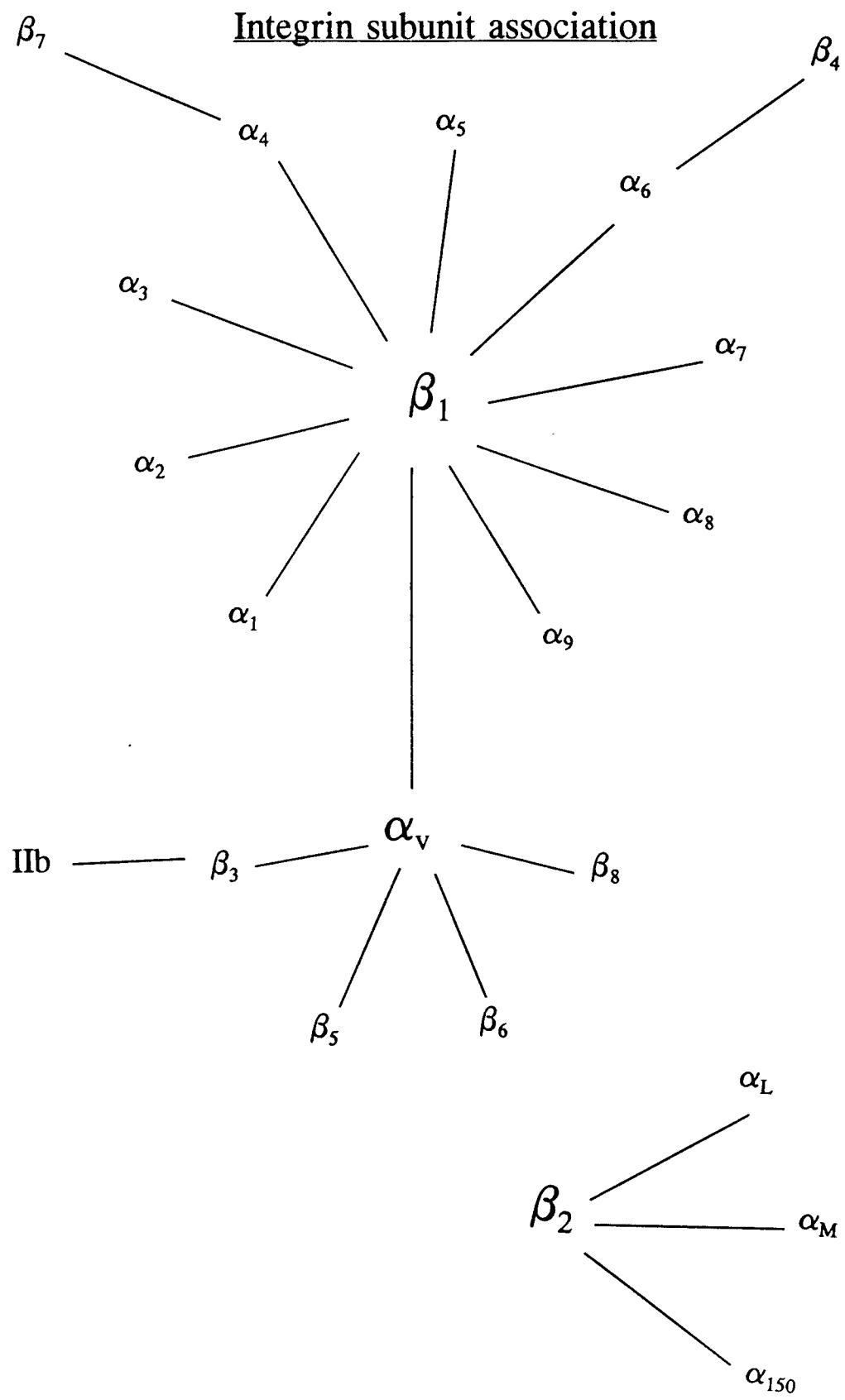
TABLE 1

Integrin superfamily of cell adhesion receptors

integrin subunits	molecular mass	ligands
$\alpha_1\beta_1$	200/110	laminin (E1), collagen
$\alpha_2\beta_1$	160/110	laminin, collagen, tenascin
$\alpha_3\beta_1$	150/110	laminin, (1,5) collagen (I, IV, VI), fibronectin, entactin
$\alpha_4\beta_1$	140/110	cell-cell, fibronectin (CS-1), VCAM-1
$\alpha_4\beta_7$	140/100	fibronectin, VCAM-1, MadCAM-1
$\alpha_5\beta_1$	155/110	fibronectin, L1
$\alpha_6\beta_1$	140/110	laminin (E8)
$\alpha_6\beta_4$	140/210	laminin 1,5
$\alpha_7\beta_1$	125/110	laminin (E8) fibronectin L-14 L-14 Lectin
$\alpha_8\beta_1$	160/110	fibronectin, tenascin, vitronectin
$\alpha_9\beta_1$	140/110	tenascin
$\alpha_L\beta_2$	170/90	ICAM-1, ICAM-2, ICAM-3
$\alpha_M\beta_2$	165/90	C3bi, fibrinogen
$\alpha_{150}\beta_2$	145/90	
$\alpha_v\beta_1$	160/110	fibrinogen, fibronectin, vitronectin, osteopontin
$\alpha_v\beta_8$	160/110	fibronectin, collagen
$\alpha_v\beta_5$	160/100	fibronectin, vitronectin, osteopontin
$\alpha_v\beta_6$	160/105	fibronectin, vitronectin, tenascin
$\alpha_v\beta_3$	160/105	laminin, Von Willebrand factor vitronectin, fibronectin, fibrinogen, osteopontin, thrombospondin, tenascin PCAM-1, (L1)-CAM,
$\alpha_{IIb}\beta_3$	140/105	fibrinogen, fibronectin, vitronectin, Von Willebrand factor, thrombospondin

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B) <u>Distribution of integrins on normal and transformed cells</u>

In general, integrins are found on almost all cell types, including fibroblasts, leukocytes, platelets, myocytes, endothelial cells and most epithelial cells. Each cell type expresses a unique pattern of integrins. Different integrins tend to be expressed depending on whether the cell is resting on a basement membrane or imbedded in connective tissue. For example, fibroblasts express higher levels of the FN receptor $\alpha_5\beta_1$ while epithelial cells express higher levels of the LM receptor $\alpha_6\beta_1$ (Tang and Honn, 1995). Similar to ECM expression, integrin expression is a dynamic process. It has been demonstrated that integrin expression is transiently altered during wound repair. Integrin expression is often reduced in transformed cells however there are few predictable changes based on the current understanding of integrin regulation. There are a few alterations in integrin expression which apply to many cells when undergoing transformation. A decrease in the expression of the $\alpha_5\beta_1$ receptor on fibroblasts and an increase in the $\alpha_v\beta_3$ are commonly observed on melanoma cells (Seftoe *et al.*, 1992).

Table 1 illustrates that most integrin receptors can bind to several ligands. Unlike many integrin receptors, the $\alpha_5\beta_1$ integrin binds primarily one ligand (FN), however, it is not without important cellular functions such as adhesion, protease production in fibroblasts (Werb *et al.*, 1989) matrix assembly (Wu *et al.*, 1993; Fogerty *et al.*, 1990), cytokine production in human chrondrocytes (Yonezawa *et al.*, 1996), cell growth in colon carcinoma cell lines (Varner *et al.*, 1995), cell survival in CHO cells (Zhang *et al.*, 1995) proliferation (Mortarini *et al.*, 1992) and differentiation (Giancotti and Ruoslahti, 1990; Adams and Watt, 1990). Expression of $\alpha_5\beta_1$ in fibroblasts is regulated by adhesion. Fibroblasts in suspension quickly reduce surface expression of $\alpha_5\beta_1$ (Dalton *et al.*, 1992).

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In addition, it is lost from the surface of keratinocytes prior to terminal differentiation but does not appear to be involved in this process (Hotchin *et al.*, 1993).

The $\alpha_2\beta_1$ integrin receptor binds primarily to ColI and LM. Its affinity for either substrate varies between cell lines. Endothelial cells use $\alpha_2\beta_1$ primarily as a LM receptor (Languino *et al.*, 1989). Platelets use $\alpha_2\beta_1$ primarily as a ColI receptor (Kirchhofer *et al.*, 1990). Most other cell types bind to both ColI and LM. Zutter and Santoro (1990) have demonstrated that $\alpha_2\beta_1$ was widely distributed using immunohistochemical techniques. It was expressed on fibroblasts, endothelial cells, and epithelial cells including skin, tonsil, breast, sweat gland, gastrointestinal tract, lung, bladder, cervix, prostate and Schwann cells. Zutter and Santoro (1990) also found $\alpha_2\beta_1$ to be more abundant in rapidly proliferating epithelium suggesting that it may play a role in regulation of proliferation. In addition to promoting adhesion and migration, the $\alpha_2\beta_1$ integrin has been well studied for its ability to cause ColI gel contraction by fibroblasts (Schiro *et al.*, 1991; Klein *et al.*, 1991).

TGF-β can induce collagen gel contraction in the osteogenic lines MG63 and HOS-MNNG by increased $\alpha_2\beta_1$ expression at the cell surface. The $\alpha_2\beta_1$ is also reported to up-regulate collagenase and collagen $\alpha 1$ (I) gene expression in osteogenic lines (Riikonen *et al.*, 1995a and b) and to be involved in accumulation of GTP-bound Ras after Ab ligation in Jurkat T cells (Kapron-Bras *et al.*, 1993). These results show that the $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrin receptors influence a number of cellular processes.

C) <u>Integrin expression on normal and neoplastic mammary cells</u>

Breast epithelial cells, like many other epithelial cells, express a number of integrin

receptors at the cell surface. Normal mammary cells typically express the $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ integrins. The $\alpha_5\beta_1$ integrin is present on myoepithelial cells but weak on luminal cells (Gui et al., 1995b). As with other tumor cells, the integrin expression is reduced on transformed mammary cells (Restucci et al., 1995; Pignatelli et al., 1991; Zutter et al., 1990). The role of the $\alpha_5\beta_1$ receptor on mammary carcinomas has not been as well studied as the $\alpha_2\beta_1$ receptor. In fact, the FN receptor, $\alpha_5\beta_1$, has not been investigated in several key studies examining the expression of integrins on mammary carcinomas using immunohistochemical techniques (Berdichevsky et al., 1994; Alford and Taylor-Papadimitriou, 1996). However, $\alpha_5\beta_1$ is expressed on several mammary carcinoma cell lines but its role is unclear (Tawil et al., 1996; Song et al., 1995). The mammary carcinoma cell lines TA3/st and TA3/Ha used the $\alpha_5\beta_1$ and $\alpha_6\beta_4$ integrins respectively to bind to hepatocytes suggesting a role for these receptors in metastasis (Kemperman et al., 1994).

Murine breast carcinoma cells showed increased expression of $\alpha_2\beta_1$ which is correlated with a decrease in the malignant phenotype and increased morphogenesis in collagen gels (Zutter et al., 1995; Berdichevsky et al., 1992). Transfection of human mammary luminal epithelial cells MTSUI-7, SKBr3 with c-ErbB2 show a reduced levels of $\alpha_2\beta_1$ and poor morphogenic ability (D'Souza et al., 1993). It was later found that c-ErbB2 downregulation of $\alpha_2\beta_1$ occurs through regulation of the transcription factor Sp1 binding to the promoter region of the α_2 gene (Ye et al., 1996).

The ligand specificity of $\alpha_2\beta_1$ integrin i.e. its function as a LM receptor, may be regulated during the malignant progression of breast cancer cells. A reduced contribution of $\alpha_2\beta_1$ integrin to the cellular LM binding appears to be associated with an increased

malignant phenotype and with epithelial-mesenchymal transition (Maemura *et al.*, 1995).

D) <u>Focal adhesions are complex structures</u>

Focal adhesions, also called adhesion plaques or focal contacts, are a localized concentration of proteins at points of adhesion in areas where the extracellular and intracellular environment are linked by transmembrane proteins. Integrins are the major transmembrane receptors found at focal adhesions. Many cell types including fibroblasts, epithelial cell, endothelial cells grown in tissue culture and platelets adhere tightly to the underlying substrate through discrete regions of the plasma membrane (reviewed in Jockusch *et al.*, 1995).

Focal adhesions are not static but highly dynamic structures that exist for a limited period of time and are tightly controlled by a finely balanced equilibrium of their components. There are 3 examples of structures *in vivo* which resemble focal adhesions (1) smooth muscle cells, (2) myotendinous junctions and (3) plasma membrane-ECM interaction of epithelial cells.

There are now approximately 50 proteins which have been localized to focal adhesions. Experiments performed using antibodies or ligand coated beads demonstrated that some proteins are activated upon integrin clustering but not ligation and more proteins cluster to focal adhesions when integrins are both ligated and clustered (Miyamoto *et al.*, 1995a and b). An interesting feature of focal adhesions is that they are enriched with a large number of proteins that are tyrosine phosphorylated. Furthermore, the assembly of focal adhesions is disrupted by inhibitors of protein tyrosine kinases and stimulated by inhibitors of protein tyrosine phosphatases (Craig and Johnson, 1996). Focal adhesions

are sites from which signal transduction pathways are generated. There are two components to the signaling mechanisms of focal adhesions; (1) mechanical components involving the cytoskeleton and changes in cell shape and (2) enzymatic components involving activation of protein tyrosine kinases. Integrin ligation and clustering to focal adhesions will cause a cascade of structural proteins to be assembled including talin, α-actinin, paxillin, tensin. The function of some proteins in focal adhesions are not yet clearly defined. However, two important tyrosine kinases at focal adhesion which are well characterized are c-Src and focal adhesion kinase (FAK). Focal adhesion kinase (pp125FAK) is a 125 kDa cytoplasmic protein tyrosine kinase which co-localizes to focal adhesion after integrin activation. Structurally FAK contains a central catalytic domain. The amino-terminal domain contains a region capable of binding the membrane proximal 13 amino acids of the $\beta1$ integrin and to c-Src. The carboxyl-terminal region of FAK is involved in targeting to focal adhesion and binding to a number of cytoskeletal proteins. Focal adhesion kinase was originally isolated as a substrate for c-Src. There are currently at least 9 FAK-binding proteins. Among these are cytoskeletal proteins such as paxillin and talin as well as proteins involved in signaling such as Grb2, Phosphatidylinositol 3-kinase (PI 3-kinase), c-Src and Cas (reviewed in Schaller, 1996).

Focal adhesion kinase is expressed in most cell types and its expression was shown to be increased in a number of metastatic breast and colon carcinomas *in vivo* (Weiner *et al.*, 1993) as well as in a number of tumor cell lines (Owens *et al.*, 1995).

E) Integrin signal transduction

The field of integrin signal transduction has exploded in the last couple of years and

an in depth review is beyond the scope of this introduction. There are several excellent recent reviews on integrin signal transduction (Lafrenie and Yamada, 1996; Schwartz *et al.*, 1995; Dedhar and Hannigan, 1996; Clark and Brugge, 1995; Guan and Chen, 1996; Yamada and Miyamoto, 1995).

Integrin signal transduction pathways are involved in a number of biological processes including cell adhesion and spreading, proliferation, apoptosis, differentiation and gene transcription. As noted previously there are approximately 21 different integrins heterodimers each of which can bind several ligands and are important for a large number of biological processes and cellular functions. This situation obviously leads to a very complex system of signaling events which are only beginning to be illucidated.

Integrins are involved in bidirectional signaling, meaning that there are both outside-in and inside-out signal transduction mechanisms. Inside-out signaling has been well studied in white blood cells and platelets. The gpIIaIIIb receptor on platelets and the β_1 and β_2 integrins on hematopoietic cells are usually unable to bind ligand until activated by other factors such as thrombin, therefore intracellular signaling is required to activate the integrin. Transfection of β_2 integrins in epithelial cells results in constitutive activation of the integrin suggesting that the constitutive activity of integrins on epithelial cells is mediated by cytoplasmic components and not the integrin receptor itself (Larson *et al.*, 1990). The identity of this mechanism is unknown but may involve the Ras proteins which can activate integrin receptors (Zhang *et al.*, 1996).

Outside-in signaling is the better studied mechanism of signaling in fibroblasts and epithelial cells. It involves ligation of the integrin to its ligand which activates intracellular proteins. In culture, ligation of integrins also activates a number of signaling molecules

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and events including activation of the Na/H$^+$ antiporter which is activated via the $\alpha_5\beta_1$ integrin regardless of cell shape (Schwartz $et\ al.$, 1991), increases in intracellular calcium, increased activation of PKC and MAP kinase (Chen $et\ al.$, 1994; Zhu $et\ al.$, 1995).

Several years ago it was thought that since integrins had very small cytoplasmic domains with no kinase region that they function mainly as receptors to the ECM generating mechanical tension and causing cell spreading. There is now plenty of evidence supporting the role of the cytoplasmic domain in outside-in signaling as well as in inside-out signaling in hematopoietic cells (see above) (O'Toole $et\ al.$, 1994; Akiyama $et\ al.$, 1994). In addition, the cytoplasmic domain confers integrin specific signals. Experiments performed where the cytoplasmic domain of the α_2 subunit is replaced with the cytoplasmic domain of α_4 or α_5 resulted in differences in the cells' ability to mediate CoII gel contraction and migration (Chan $et\ al.$, 1992). There is also a sequence in the cytoplasmic domain of the β_1 subunit which is responsible for targeting to focal adhesions (Marcantonio $et\ al.$, 1990). The cytoplasmic domain of the β_4 subunit is linked to pathways which are involved in cell cycle regulation since it could induce expression of p21 (WAF1) protein, an inhibitor of cyclin dependent kinases (Clarke $et\ al.$, 1995).

The integrin subunits α_3, α_6, α_7, β_1, β_3 and β_4 have alternately spliced variants in their cytoplasmic domain. The β_1 subunit now has 4 different alternatively spliced isoforms in the cytoplasmic domain. The β_{1D} isoform is primarily located in the junctional structures in striated muscles (Belkin $et\ al.$, 1996). The β_{1C} does not colocalize to focal adhesions and when transfected in fibroblasts markedly inhibits DNA synthesis and causes growth arrest at the late G_1 phase of the cell cycle (Meredith $et\ al.$, 1995). The α_6 subunit contains 2 alternatively spliced isoforms. The α_{6A} isoform was more efficient than the α_{6B}

isoform in inducing tyrosine phosphorylation of several cytoplasmic protein in the P388D1 macrophage cell line (Shaw *et al.*, 1995).

The integrin cytoplasmic domains contain no SH2 or SH3 domains and have no kinase activity and were thought not to be involved in signal transduction. However, there are a number of proteins which have been demonstrated to bind to the integrin cytoplasmic domain including cytoskeletal components such as α-actinin (Otey *et al.*, 1990) and talin and enzymes such as ILK (integrin linked kinase) (Hannigan *et al.*, 1996), FAK, calreticulin (Rojiani *et al.*, 1991), β_3-endonexin (Shattil *et al.*, 1995), Shc and Grb2 bind to the β_4 cytoplasmic domain (Mainiero *et al.*, 1995).

There have been a number of different proteins shown to be involved in integrin signaling, including kinases such as FAK, Src, Csk, PKC and MAP kinase, SH2-SH3 signaling proteins such as Crk, Grb2, PI 3-kinase, and PLC, small molecular weight GTPase such as ras, Rho C3G and phospholipid mediators such as PIP 5-kinase, cPLA$_2$ and arachidonic acid (Clark and Brugge, 1995) have all been implicated in integrin signal transduction pathways.

F) <u>Cooperative interactions between different integrin receptors</u>

A new area in integrin research which has recently developed is the cooperative interactions between integrins. There are several reports of 2 integrins types working in a positive or negative manner in cooperative interactions. Huhtala *et al.*, (1995) showed that $\alpha_4\beta_1$ was required to downregulate MMP expression of rabbit synovial fibroblasts plated on 120 kDa fragments of FN. CHO cells required the FN receptor $\alpha_5\beta_1$ to work in cooperation with VN receptors for haptotactic response on VN since α_5 deficient cells do

not migrate on VN (Bauer *et al.*, 1992). Melanoma cells which express the VN and fibrinogen receptor $\alpha_v\beta_1$ cooperate with $\alpha_5\beta_1$ to mediate adhesion and spreading on fibronectin (Marshall *et al.*, 1995). Blystone *et al.*, (1994) found that ligation of $\alpha_v\beta_3$ could inhibit $\alpha_5\beta_1$ mediated phagocytosis in K562 cells but not $\alpha_5\beta_1$ mediated adhesion. This inhibition could be blocked using the serine/threonine kinase inhibitor H7. In addition Charo *et al.*, (1990) showed that $\alpha_v\beta_3$ acted cooperatively with the $\alpha_5\beta_1$ receptor to mediate adhesion and spreading in M21 melanoma cells. These results show that integrins can act together in a cooperative or negative manner and show another level of complexity in integrin regulation.

1.3 - GROWTH FACTORS

Growth factors are polypeptides which can stimulate processes such as growth, proliferation, differentiation, migration and survival of cells. They mediate their effects through high affinity interactions with transmembrane cell surface receptors that modify key regulatory proteins in the cytoplasm. *In vivo*, normal cells typically respond to growth factors in a paracrine manner. In contrast, transformed cells frequently become independent of exogenous growth factors and acquire an autocrine loop where they respond to growth factors to which they have secreted.

A) Hepatocyte growth factor

Hepatocyte growth factor (HGF) also called scatter factor belongs to the family of kringle proteins, characterized by triple disulfide loop structures (kringles) that mediate protein-protein and protein-cell interactions. HGF is a mesenchymal-derived heparin-

binding glycoprotein that is secreted as a single-chain (pro-HGF), biologically inert precursor. proHGF can then be cleaved by enzymes such as urokinase plasminogen activator or by a protease homologous to factor XII. It then acts predominantly on cells of epithelial origin in an endocrine and/or paracrine fashion. Mature HGF is a heterodimer, consisting of a 60 kDa α and 30 kDa β chain linked by a single disulfide bond.

HGF is also involved in a number of normal developmental pathways such as proliferation, migration, liver regeneration, mammary gland morphogenesis, invasion and tumor progression (Rosen *et al.*, 1994). HGF regulates growth and motility of a number of epithelial cell lines (Jiang *et al.*, 1993) as well as anchorage-independent growth of MDCK cells (Uehara and Kitamura, 1992). Growth, morphogenesis, branching and during development of mammary cells are also dependent on HGF (Niranjan *et al.*, 1995; Soriano *et al.*, 1995). Transfections of HGF into immortalized liver cells transformed them into tumorigenic cells (Kanda *et al.*, 1993). HGF can also promote survival of adherent rat pheochromocytoma PC12 cells in low serum condition (Matsumoto *et al.*, 1995a).

HGF and c-Met are expressed and regulated temporally during mouse mammary development and differentiation. It is both mitogenic and morphogenic for mammary epithelial cells in collagen gels (Niranjan *et al.*, 1995; Soriano *et al.*, 1995) and also causes branching of MDCK cells in collagen gels. The presence of other ECM proteins such as LM, entactin, and FN facilitated the branching and morphogenesis while others like type IV collagen, heparan sulfate proteoglycan and VN inhibited HGF-mediated morphogenesis (Santos and Nigam, 1993). In another study Clark (1994) showed that MDCK cells

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scattered in response to HGF only on FN coated surfaces and not on VN coated surfaces, however, they observed no difference in the mitogenic response on the two substrates. HGF could also promote scatter activity of several colon carcinoma cell lines but inhibited their growth over a period of several days (Jiang *et al.*, 1993). In relation to HGF mediated motility, HGF could induce tyrosine phosphorylation of FAK in oral squamous carcinoma cells (Matsumoto et al., 1994). HGF could induce anchorage-independent growth of Mv1Lu cells in agar while 10% FBS alone or with TGF-β could not (Taipale and Keski-Oja, 1996). Transfection of HGF in MDCK cells caused an increase in colony growth (Uehara and Kitamura, 1992). Addition of exogenous HGF could increase anchorage-independent growth of small cell lung cancer cells (Seckl *et al.*, 1994).

TGF-β inhibited branching and tubulogenesis of MDCK cells in collagen type I gels (Santos and Nigam, 1993). HGF could also partially inhibit TGF-β mediated growth arrest of Mv1Lu cells (Taipale and Keski-Oja, 1996).

B) HGF receptor (c-Met)

The c-Met receptor is a 190 kDa glycoprotein consisting of a 145 kDa membrane spanning β-chain and a 50 kDa α-chain. The extracellular binding domain and tyrosine kinase domain are located in the β-chain. Two other receptors, c-Sea and c-Ron are members of this family however their ligands remain unknown. The c-Met receptor possesses a multifunctional binding site involving two YV(H/N)V motifs which mediate binding to PI 3-kinase, protein tyrosine phosphatase 2, PLC-γ, c-Src, and Grb2/Sos via tyrosine residues (Ponzetto *et al.*, 1994).

C-Met is expressed in normal epithelium of almost every tissue, however, other cell

types such as melanocytes, endothelial cells, microglial cells, neurons and hematopoietic cells also express c-Met. In addition, c-Met is expressed in most carcinoma cells in culture and is expressed at very low levels in human fibroblasts. The demonstration of co-expression of HGF and c-Met in human bronchial epithelial cells suggested that an autocrine loop may play a regulatory role during the development and growth of human bronchi (Byers et al., 1994).

All of the biological effects of HGF/SF are transduced through its cellular receptor, the product of the c-Met proto-oncogene, by the activation of its tyrosine kinase domain and the phosphorylation of a multifunctional docking site, which is responsible for the engagement of downstream mediators. Since tumor malignancy depends on events such as the ability of cancer cells to undergo uncontrolled cell proliferation, loss of differentiation, acquisition of a motile and invasive phenotype and induction of neovascularization, the untimely activation of the c-Met receptor might give a strong contribution to the metastatic phenotype.

C) Transforming Growth Factor-β and its receptors

TGF-β's are potent regulators of cellular differentiation, morphogenesis, proliferation and growth. TGF-β interacts primarily with the protein core of proteoglycans. In addition to being bound by ECM, TGF-β1 modulates many aspects of matrix biochemistry (Yamagouchi et al., 1990). TGF-β regulates the composition of ECM by stimulating the synthesis of ColI, FN, tenascin, proteoglycans, and integrins, acting to inhibit ECM degradation in some cells through down-regulation of proteinases such as plasminogen activators, collagenase, and stromelysin; and by up-regulating proteinase

inhibitors such as the tissue inhibitor of metalloproteinase 1 and PAI-1 (Sehgal *et al.*, 1996). TGF-β also induces the expression of specific matrix proteoglycans such as decorin and biglycan. This stimulation is of particular significance because these proteoglycans bind TGF-β1 and appear to inhibit its activity. The significance of the binding of TGF-β binding to FN, thrombospondin, Coll IV or decorin is less apparent (Yamagouchi *et al.*, 1990).

TGF-β is a 25 kDa disulfide linked homodimer which belongs to a family of structurally related polypeptides that regulate cell growth and differentiation. TGF-β's are ususally synthesized and secreted in a latent form. They can be activated by a number of mechanisms including pH extremes, proteases, thrombospondin, irradiation, glucocorticoids and retinoids (Koli and Keski-Oja, 1996).

TGF-β is a heat- and acid-stable peptide composed of two identical chains held together by disulfide bonds. Activated TGF-β1 has multiple reported biological effects. It is a potent regulator of cell proliferation, acting to stimulate the growth of mesenchymal cells and inhibit the growth of other cell types by inducing a block in G1. Meth A sarcoma cells, which overproduced TGF-β1 demonstrated growth inhibition *in vitro*, but were more tumorigenic *in vivo* as assayed by increased tumor size and number. Studies in rat prostate cancer cells have also shown that transfection with a TGF-β1 expression vector resulted in TGF-β1 overproduction and led to transient growth inhibition *in vitro*, but resulted in larger tumors with a greater number of metastases after either i.v. or local deposition *in vivo* (Sehgal *et al.*, 1996).

TGF-β is also involved in the development of the mammary gland. Mammary epithelial cells in culture, as well as several breast carcinoma cell lines, express TGF-β and

are responsive to its growth inhibitory effects. In the later stages of tumor development, cells acquire hormone independence following progression towards estrogen independence there is a large increase in TGF-β production and change in cellular responses towards this growth factor. TGF-β mRNA is usually upregulated in breast cancer (Walker *et al.*, 1994; Welch *et al.*, 1990).

TGF-β receptors initiate signaling pathway with serine/threonine kinase activity. The cellular growth response to TGF-β is also dependent on the differentiation and activation state of cells and on the presence of other extracellular growth regulatory molecules (Koli and Keski-Oja, 1996). One of the three species of cell-surface receptors with which TGF-β associates is a 250-350 kDa heparin and chondroitin sulfate proteoglycan called betaglycan which is present as soluble and matrix bound species.

TGF-β1 binds to three types of cell surface receptors. Type I TGF-β receptors contain a serine-threonine kinase domain and have been reported to transduce signals which promote the synthesis of matrix proteins; the type II receptor, also a serine-threonine kinase, is reported to mediate antiproliferative effects, and the type III receptor or β-glycan is a proteoglycan which binds TGF-β1 but does not transduce growth factor signals. Heterodimerization of type I and type II receptors is required for TGF-β1 signaling through either receptor, although the type II receptor can directly bind TGF-β ligands (Sehgal *et al.*, 1996).

D) Receptor tyrosine kinase signal transduction and PI 3-kinase

Growth factor receptor tyrosine kinases (RTK) possess an extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic region with inducible

protein tyrosine kinase activity. Receptor ligation results in activation and association of the receptor with a number of cytoplasmic signaling proteins, that in turn stimulate intracellular signal transduction pathways and induce changes in cellular gene expression, cytoskeletal architecture, proliferation, migration and metabolism.

Receptor-ligand interactions cause dimerization of the receptors tyrosine kinase which physically associate with and phosphorylate a set of cytoplasmic proteins implicated in cellular signal transduction pathways. These include phospholipase C-γ (PLC-γ), phosphatidylinositol 3-kinase (PI 3-kinase), p21 Ras GTPase activating protein (GAP) and members of the Src tyrosine kinase family. A detailed review of RTK signaling is beyond the scope of this introduction, I will focus on PI 3-kinase signaling.

PI 3-kinase is a dual specificity enzyme involved in a number of cellular functions including mitogenesis, apoptosis, vesicle trafficking and cytoskeletal assembly. PI 3-kinase can affect cell growth in three ways (1) through the production of phosphoinositides (2) through phosphorylation of proteins on serine residues via the endogenous protein kinase activity, or (3) by acting as an adaptor protein, not requiring enzymatic activity (Carpenter and Cantley, 1996). Yao and Cooper, (1995) have shown that PI 3-kinase is required for nerve growth factor (NGF) mediated survival of PC12 cells and rat fibroblasts (Yao and Cooper, 1996). In addition PI 3-kinase activity was required for FAK activation by PDGF in human umbilical vein smooth muscle cells. Mutant PDGF receptors unable to bind PI 3-kinase were unable to increase FAK phosphorylation in response to PDGF (Varticovski *et al.*, 1994).

Phosphatidylinositol 3-kinase (PI 3-kinase) is composed of an 85 kDa (p85) regulatory subunit and a 100 kDa (p110) catalytic subunit. The p85 subunit has two

isoforms (α and β) which have extensive amino acid homology. The p85α isoform is ubiquitously distributed, whereas the p85β isoform is primarily found in bovine brain and lymphoid tissues. The primary sequence of p85 consists of several domains including one SH3(mediates protein-protein interactions via proline rich regions), two SH2 (mediates association with both receptors and non-receptor tyrosine kinases via phosphorylated tyrosine residues) and a BCR-like domain who's function in PI 3-kinase is unknown but in other proteins mediates GTPase activity for small GTP binding proteins.

PI 3-kinase associates with a number of proteins via both its SH2 and SH3 domains. In addition to growth factor receptors, the SH2 domain of p85 binds to src, rasGAP, PLC-γ, Crk, Grb2, Shc and FAK. The two SH2 domains bind tyrosine phosphorylated receptors and in this manner recruit the p85-110 complex to activated receptors. The two SH2 domains are separated by an inter-SH2 region which mediates the interaction of p85-110. This interaction is required for the enzymatic activity of p110. The catalytic p110 subunit is homologous to other protein kinases and, itself, has protein serine/thronine kinase activity, as well as phophoinositide kinase activity (Carpenter and Cantley, 1996). In addition the SH3domain of c-Abl, Lck, Fyn, Lyn, Blk, and Src can bind to the proline rich region of PI 3-kinase. The principal reaction catalyzed by PI 3-kinase is the phosphorylation of PI, PI 4-P and PI 4,5-P_2 to form PI 3-P, PI 3,4-P_2 and PI 3,4,5-P_3 respectively (Varticovski *et al.*, 1994).

E) <u>Cooperative signaling between integrins and growth factors</u>

There is overlap between receptor tyrosine kinase and integrin signal transduction pathways. It appears that there may be additional, adhesion specific events involved in

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mitogenesis, because high concentrations of growth factors are not sufficient to override the adhesion requirement (Kang and Krauss, 1996). However, the molecular mechanisms which join integrin and growth factor signaling pathways are not well understood. There are a number of ways in which ECM and growth factors act co-operatively. (1) The ECM presents bound growth factors to cells in a functional manner (2) the ECM may change the cell shape through adhesive interactions and render it responsive to growth factors (3) ECM proteins may bind to integrins and generate signals that interact with those generated by growth factors (Schubert, 1992). One function of the ECM which is important for growth factor signaling is the activation of PIP 5-kinase catalyzed PIP_2 formation by adhesion to FN which is ready to be used in growth factor signaling pathways (McNamee *et al.*, 1993).

There are a number of signaling molecules which are well established in the growth factor signaling pathways which are now being demonstrated to be involved in integrin signaling pathways. For example Ras, Grb2, Src and FAK have been implicated in such signaling pathways. These results suggest cooperative or synergistic interactions between growth factors and integrin signal transduction pathways.

Until recently the effect of adhesion via integrins and the effects of growth factor on cellular proliferation and other functions were studied independently. Most studies performed on the cooperative effect between integrins and growth factors have been done in fibroblasts and platelets. Little information is available in this area on epithelial cells.

Src, an established member of growth factor signal transduction, is also colocalized at focal adhesions during the initial stages of adhesion. $Src^{-/-}$ fibroblasts demonstrated slower adhesion kinetics to FN than wildtype cells. The kinase activity of Src was not

required for cell spreading (Kaplan *et al.*, 1995).

FAK, a key player in integrin signal transduction is also phosphorylated in response to growth factor stimulation (Matsumoto *et al.*, 1994). FAK phosphrylation after growth factor activation may be one of the mechanisms by which growth factors regulate cell adhesion. Another protein which may be invovlved in altering adhesion via growth factors signaling is the small GTP-binding protein rho, which regulates focal adhesion assembly and stress fibres (Ridley and Hall, 1992).

1.4 - ANCHORAGE-INDEPENDENT GROWTH

Anchorage-independent growth is defined as growth without adhesion and spreading and is considered a characteristic of most tumor cells. The term anchorage-independent growth refers to growth and proliferation in the absence of adhesion and spreading. The classical assay to determine the anchorage-independent potential of cells is growth in soft agar. Cells which are able to grow in soft agar generally are more tumorigenic in mice (Shin *et al.*, 1975). These properties suggest a relationship between the degree of transformation and the anchorage-independent growth potential. The term anchorage-independence was coined many years ago before the understanding of cell adhesion and integrins. We, in this thesis, and others (Glimelius *et al.*, 1988; Nederman *et al.*, 1984) have shown the involvement of integrins and ECM in cells which are not adherent or spread, which conflicts with the original term anchorage-independence. From herein, the term "anchorage-independence" will refer to spreading independence or growth in a three dimensional matrix. In our survival assay the cells are maintained in suspension

in media while they are inbedded in a three-dimensional agar matrix in the colony assays. Non-transformed cells must be adherent and spread in order to proliferate. As the cells progressively become transformed there is a progressive loss of anchorage requirement and response to exogenous growth factors via paracrine stimulation.

The most common method used to determine anchorage-independent growth is growth in soft agar. Tumor nodules have been shown to be more resistant to chemotherapy and radiation therapy than adherent cells. A tumor spheroid typically shows several layers of cells with a necrotic center, several layers of cells undergoing apoptosis, few layers of quiescent cells and an outer layer of rapidly proliferating cells. It is likely the layer of quiescent cells which are resistant to conventional treatments (Olive and Durand, 1994). As the spheroids enlarge, the external well-nourished cells continue to divide but the internal cells, lacking nutrients and O_2 often exit the cell cycle and enter G_0. It is these cells which have exited the cell cycle which are likely to be resistant to chemotherapy and radiotherapy since these treatments are designed for rapidly dividing cells.

No correlation was found in a survey of the anchorage-independent growth of epidermal keratinocyte and human squamous cell carcinoma cell lines and oncogene expression (Kim *et al.*, 1991). In addition transformation of rat embryo fibroblasts with v-Src transformed 95% of the cells but only 25% were anchorage-independent (Tavoloni *et al.*, 1994). Despite these studies, oncogenic transformation can render some cells anchorage-independent (Masuda *et al.*, 1992; Rak *et al.*, 1995).

Another common three-dimensional culture model used is collagen gels where cells are embedded in a Coll I matrix. This system has been used primarily to study the role of

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Coll receptors and Coll gel contraction.

A) ECM and integrins in anchorage-independent growth

There is limited information on the role of ECM proteins and integrins in anchorage-independent growth of either normal or transformed cells. The presence of ECM protein such as FN, LM, collagen and proteoglycans in tumor spheroids has been demonstrated many years ago in glioma cell lines (Glimelius *et al.*, 1988; Nederman *et al.*, 1984; De Pauw-Gillet *et al.*, 1988). More recently, bovine granuloma cells cultured in agar produced FN and Coll IV (Rodgers *et al.*, 1995).

There were several studies performed on the expression of integrins in tumor spheroids. The α_6 and β_1 subunits were decreased in tumor spheroids of squamous cells A431, while there were no changes in the α_2, α_5, β_4 (Waleh *et al.*, 1994). Hauptmann *et al.*, (1995) investigated the expression of integrins on colorectal cells growing either as monolayers or spheroids *in vitro*, or as tumors and *in vivo* tumors in nude mice. They showed that different integrins were either decreased or increased in the different culture conditions and concluded that the different microenvironments regulated integrin expression. In addition, fibroblasts typically downregulated both FN and $\alpha_5\beta_1$ expression when maintained in suspension (Dalton *et al.*, 1992).

B) Growth factors and anchorage-independent growth

Growth factors are capable of driving adherent cells through the cell cycle. However, normal cells such as 3T3 L1 or NRK fibroblasts will not progress through the cell cycle, in the presence of growth factors, if maintained in suspension. Transformed

cells have acquired growth factor independence through autocrine secretion of growth factors, mutations or unregulated expression in growth factor receptors or proteins involved in signal transduction, both of which cause constitutive activation. The role of several growth factors in anchorage-independent growth has been investigated. Growth factors such as EGF, FGF and PDGF can increase anchorage-independent growth of some cells in the presence of 10% serum (Rizzino et al., 1986). TGF-β is well documented in the literature as being able to increase anchorage-independent growth of a number of cell lines. Interestingly TGF-β is usually a growth inhibitor when cells are adherent to ECM. It is clear that TGF-β affects the expression of both ECM proteins such as FN, as well as their corresponding integrin receptors. It is not known what effect TGF-β has on ECM and integrin expression on anchorage-independent cells.

C) The cell cycle and anchorage-independent growth

Untransformed cells, with the exception of hematopoietic cells, typically require stimulation from both adhesion and growth factors to progress through the cell cycle (Guadagno et al., 1993; Han et al., 1993). Adherent cells irreversibly commit to cell cycle progression at a point in late G_1 known as the restriction point (Pardee, 1989; Guadagno et al., 1991). Expression of cyclin D1 is induced by serum growth factors and is required through early G_1. In late G_1, cyclin E is synthesized and is required for entry into S phase in fibroblasts. Cyclin A gene expression starts at the G_1/S transition and is required for S and G_2 phases (Figure 3). When cultured in suspension, fibroblasts and normal NRK cells exit G_0 upon serum stimulation. They express G1 cyclins but fail to express cyclin A which is required for S phase entry. In suspended cells, the transcription

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factor E2F remains bound to the cyclin A promoter. Phosphorylation of pRb and p107 by cyclin D- and cyclin E-dependent kinases appears to be crucial to the regulation of E2F activity (Schulze *et al.*, 1996; Zhu *et al.*, 1996). Expression of cyclin A was also anchorage-dependent in human primary forskin fibroblasts. Constitutive expression of cyclin A is not sufficient to bypass the earlier growth factor dependent events that now seem to coorelate with the expression of cyclin D and E. However cyclin E-cdk2 activity is also anchorage-dependent (Fang *et al.*, 1996).

Cyclin D1 deregulation is found in many different tumor types including mammary carcinomas (Zwijsen *et al.*, 1996). Overexpression of cyclin D1 in MCF-7 accelerates growth under conditions in which growth factors are limiting but cannot render the cells completely growth factor independent.

In addition Symington (1992 and 1995) demonstrated that activation of the $\alpha_5\beta_1$ receptor on K562 cells via an RGD peptide stimulated cyclin A/cdc2 activity and increased anchorage-independent of these cells.

Figure 3. Cell cycle regulation

This Figure demonstrates the stages of the cell cycle (G1,S,G2, and M), and the location in the cell cycle where specific cyclin dependent kinases (CDK) and cyclins are active. The figure also illustrates where growth factors such as HGF and adhesion are required for progression through the cycle and where TGF-β inhibits the cell cycle.

Figure 3

CELL CYCLE REGULATION
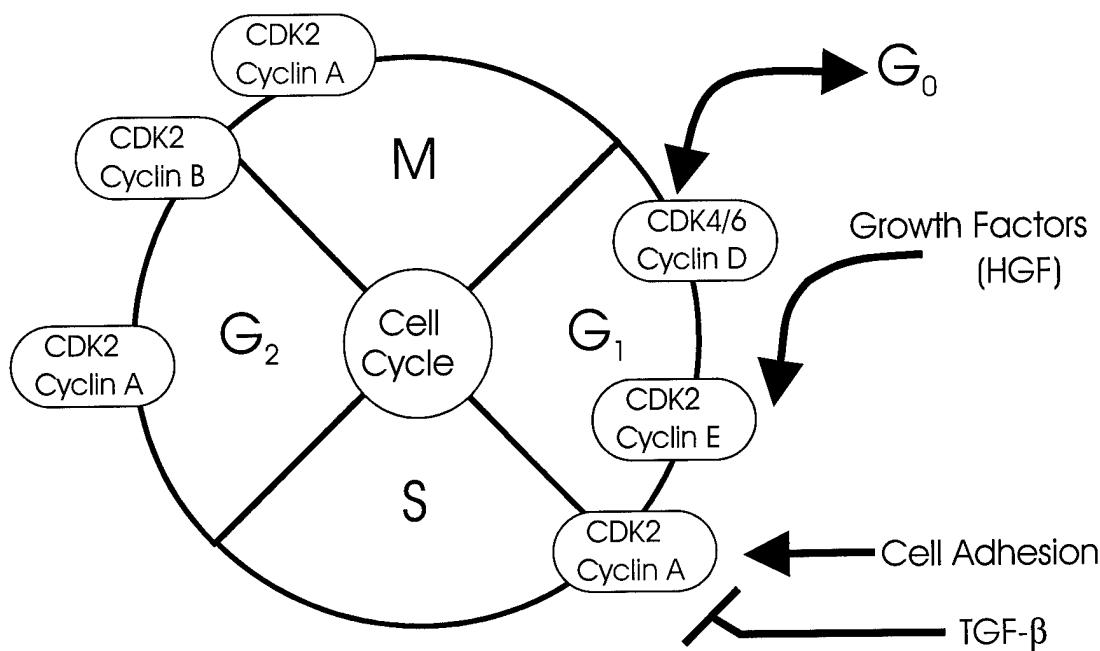
D) Adhesion and cell survival

A recent development is that integrin-mediated adhesion and signaling is required for survival. Detachment from a substrate causes the cells to undergo apoptosis. This mechanism for apoptosis has been termed anoikis (Frisch and Francis, 1994). Only integrin-mediated attachment circumvents anoikis; attachment to antibodies directed to MHC proteins or plated on a polylysine substrate, failed to rescue the cells from apoptotic death.

It has been shown that anchorage-dependent cells when denied attachment, not only stop proliferating but also undergo apoptosis (Meredith et al., 1993; Frisch and Francis, 1994). How anchorage-independance relates to integrin signaling and what role $\alpha_5\beta_1$ might play in anchorage-independent growth is yet to be resolved. CHO cells transfected with the $\alpha_5\beta_1$ integrin were able to inhibit serum deprived apoptosis on immobilized fibronectin by upregulating Bcl-2 but those transfected with $\alpha_v\beta_1$ could not inhibit apoptosis (Zhang et al.,1995). It was also found that the α_5 cytoplasmic domain was required for survival on fibronectin. Montgomery et al., (1994) have shown that ligation of the VN receptor $\alpha_v\beta_3$ within a three-dimensional dermal collagen matrix, suppresses apoptosis and promotes melanoma (M21) cell growth. CHO and osteosarcoma cells cultured in serum-free conditions, survived only if they attached through the $\alpha_5\beta_1$ integrin. Whereas some other integrins, while supporting cell attachment, could not rescue the cells from apoptosis (Zhang et al., 1995). FAK has also been implicated in the maintenance of survival since FAK knockouts in cell lines were undergoing apoptosis (Frisch et al., 1996).

In conclusion ECM-integrin interactions and cooperative signaling with growth factors play an essential role in both normal and transformed cell behaviour. The

interactions have been well studied in adherent cells but are not well understood in three-dimensional spheroids, which is an intermediate model between cells in culture and *in vivo* tumor nodules.

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

The incidence of cancer is continually on the rise (Parker *et al.*, 1997). An unfortunate circumstance of an untreated tumor is that it can metastasize to other organs via local invasion or systemically through the blood stream or lymphatic vessels. Were it not for these many metastatic nodules, the primary tumor could be surgically removed and the patient cured of his/her tumor. Once a tumor has metastasized, treatment is more difficult and almost always leads to the eventual death of the patient. These facts stress the need for better treatments and preventative methods. Before these can be accomplished, a better understanding of how tumor cells grow and interact with their microenvironment is needed.

There are several phenotypic characteristics which are common in tumor cells, including rapid proliferation, loss of differentiation, oncogene activation or tumor suppressor gene inactivation and genetic instability. *In vivo*, the tissue microenvironment is very dynamic, continually changing to meet the needs of the cell. During transformation, there is often an altered synthesis and secretion of ECM proteins as well as changes in the corresponding cell surface receptors. Extracellular factors such as ECM and growth factors have been shown to influence tumor cell growth and differentiation. In fact, Lochter and Bissell (1995) state "The ECM constitutes a structure impinging on most, if not all, master switches associated with cancer progression from a benign lesion to an aggressive invasive and thus life-threatening phenotype."

Tumor model

Tumor spheroids have been used as a tumor model for a number of years. They were shown to histologically resemble *in vivo* tumors and are more physiologically similar to *in vivo* tumors than cells cultured in monolayers. Tumor spheroids are an intermediate system between tissue culture and *in vivo* tumors. Roskelley and Bissell (1995) found that mammary cells not only required basement membrane proteins to differentiate but also required a rounded morphology. Howlett *et al.* (1995) have shown that normal mammary cells differentiate into well-organized acinar structures whereas tumor cells form large, disorganized colonies when plated in the basement membrane matrix, Matrigel.

Acquiring a better understanding of how extracellular factors such as ECM and growth factors affect metastatic cell growth in an anchorage-independent manner as found in tumor spheroids may provide the basis for developing better therapies to treat metastatic lesions.

The mammary gland is a good system to investigate epithelial cell proliferation, differentiation and tumorigenesis. Unlike other organs, the mammary gland develops to maturity in the adult animal, reaching its fully functional status only during late stages of pregnancy and lactation. Pregnancy induces a massive proliferation of the epithelial cells, which leads to a branching morphogenesis which is accompanied by the production and secretion of milk proteins by the epithelial cells. After the lactation period, the epithelium is dismantled during involution by a process that involves degradation of the ECM prior to apoptosis, suggesting that the ECM is required for survival of mammary epithelial cells (Pullan *et al.*, 1996).

The ECM has been shown to profoundly influence mammary cell function during

development, morphogenesis during pregnancy and during involution, and tumorigenesis (discussed in introduction). In addition, tumor cells frequently have altered integrin expression on the cell surface. The last couple of years has shown an explosion in the field of integrin signal transduction giving this family of molecules new life in the role of tumor cell adhesion, migration, differentiation and invasion. The integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ are important in mammary cell morphogenesis (Howlett *et al.*, 1995). However the role of $\alpha_5\beta_1$ and FN are not well understood.

In addition to ECM proteins and integrins, growth factors can play an essential role in mammary cell development and transformation. A number of growth factors, including EGF and TGF-α are important regulators of both proliferation and differentiation of mammary cells *in vivo*. After transformation, a change in the balance of growth factors such as FGF, IGF, PDGF and HGF may stimulate tumor cell proliferation. TGF-β, a potent regulator of ECM and integrin expression, is particularly interesting since this growth factor can be both inhibitory or stimulatory for cells. TGF-β is often expressed in invasive mammary carcinomas (Oda *et al.*, 1992, Travers *et al.*, 1988) and therefore may also play a role in growth of breast cancer cells (Welch *et al.*, 1990). Another important stromally derived growth factor potentially involved in mammary carcinogenesis is HGF. It is a pleiotrophic growth factor involved in morphogenesis as well as proliferation and migration of a number of cell types.

We have previously demonstrated that mammary fat tissue or 3T3-L1 preadipocyte conditioned media can stimulate the growth of a murine mammary carcinoma cell line, SP1 (Elliott *et al.*, 1988, 1992; Rahimi *et al.*, 1996a). These cells grown in soft agar contain abundant extracellular FN in the form of fibrils. Much of what is known about the effects

of ECM on mammary cells is based on differentiation and not growth. Much of what is known about cell growth under anchorage-independent conditions is based on growth factors and normal fibroblasts and concentrates on the cell cycle. *There are few studies on the role of ECM in anchorage-independent survival and growth of tumor cells.* This thesis will look primarily at the effects of ECM on anchorage-independent growth but also considers growth factors. We now want to show how extracellular matrix and growth factors participate in anchorage-independent survival and growth of SP1 cells. **Our hypothesis is that extracellular matrix proteins and growth factors stimulate their respective cell surface receptors under anchorage-independent conditions and generate intracellular signals which permit survival and colony growth of SP1 cells.**

Overall Objectives

1. To determine the adhesive properties and integrin profiles of SP1 cells.

2. To determine the effect of ECM proteins and growth factors on anchorage-independent growth of SP1 cells.

3. To isolate SP1 cell clones with high and low colony forming efficiency and to determine whether specific ECM-integrin interactions are associated with anchorage-independent growth.

4. To establish whether ECM and growth factors are involved in survival of SP1 cells under anchorage-independent conditions.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Iodine ^{125}I and ^{3}H-thymidine were purchased from Amersham. [γ-^{32}P] ATP was purchased from NEN. Western blot chemiluminesence reagents were purchased from Dupont. The Micro BCA protein Assay Reagent Kit was purchased from Pierce. Bacto-agar was purchased from Difco.

3.2 Matrix proteins, growth factors and antibodies

Bovine plasma fibronectin, rat tail collagen types I and IV, bovine vitronectin, and mouse laminin were purchased from Gibco. BSA was obtained from Sigma. All extracellular matrix proteins received lyophilized were reconstituted according to the manufacturer's specification and kept frozen at -70°C in 100 μl aliquots at a concentration of 1 mg/ml. FITC-labelled FN, and the 70 and 85 kDa fragments of FN were gifts from Dr. D. Mosher. The anti-bovine FN antibody was purchased from Telios.

The anti-α_3 and anti-α_5 polyclonal antibodies, used in the immunoprecipitation of integrins, directed to the integrin cytoplasmic domain are gifts from Martin Hemler. The α_5 antibody (5H10) was obtained from Pharmingen and is directed to the extracellular domain of the α_5 subunit. TGF-β was purchased from Sigma. HGF is from Genentech (gift from Dr. Ralph Schwall). The anti-phosphotyrosine antibody was obtained from Signal Transduction Laboratories. The polyclonal anti-β_1 antibody was a gift from Dr. K. Rubin. The secondary anti-mouse and anti-rabbit horseradish peroxidase conjugated antibodies were purchased from Amersham. The anti-PI 3-kinase and anti-FAK antibodies were purchased from Upstate Biotechnology Incorporated. The α_2 antibody and the BMA5 (directed to the

mouse α_5 subunit) was obtained from Dr. Bosco Chan. The BMA5 antibody is a rat monoclonal antibody produced from a rat-mouse hybridoma fusion. BMA5 is directed to the extracellular portion of the α_5 integrin subunit and interferes with integrin-ligand binding; hence it is a blocking antibody (Fehlner-Gardiner *et al.*, 1996). It was initially provided to us as a hybridoma supernatent containing 10% FBS. The antibody concentration was calculated by B. Chan to be approximately 1 μg/ml. It is referred to in this thesis as "hybridoma conditioned medium" or (sup). This antibody was later provided to us in a concentrated form after being purified by ammonium sulfate precipitation and protein G-sepharose affinity column. The antibody concentration was calculated by B. Chan to be approximately 500 μg/ml. This antibody is referred to in this thesis as "affinity purified" or (conc).

3.3 Cell lines

The SP1 tumor is a spontaneous non-metastatic murine mammary intraductal adenocarcinoma isolated in 1982 from an 18 month old CBA/J female retired breeder in the mouse colony at Queen's University. Subsequently, the SP1 tumor cell line was established, and characterized (Elliott et al.,1988). The established SP1 cell line was frozen at -70°C to maintain stocks.

SP1 cells and SP1 cell clones, Cl-12-H and Cl-24-L, were maintained in RPMI 1640 supplemented with 7% FBS, subcultured every two days, and were kept in culture for no longer than 3 months before thawing a fresh stock.

3.4 Cloning of SP1 cells

SP1 cells were plated at a very low dilution in 100 x 15 mm tissue culture plates in RPMI 1640 supplemented with 7% FBS. The location of single cells were marked and once small islands had grown, the cells were loosened with 5.0 mM EDTA and transferred to 24-well tissue culture plate. Once the cells were confluent they were transferred to a 100 X 15 mm plate and cultured for several passages before being assayed. Stocks were frozen in liquid nitrogen.

3.5 Cell adhesion assay

Linbro 96 well-tissue culture plates were precoated with a two-fold serial dilution of ECM proteins starting at 20 μg/ml of FN, LM, VN, Coll I, and Coll IV at 4°C overnight. In some experiments the plates were coated with 10 μg/ml of the specified ECM protein. The following day the wells were washed 3 times with PBS and blocked with 0.5 mg/ml BSA in RPMI 1640 for 2 h at 37°C. Cells were harvested and resuspended in RPMI 1640 containing 0.5 mg/ml BSA. SP1 cells (30,000/well) were plated and incubated at 37°C for 45 min. This number of cells is sufficient to cover the entire surface of the well without an excess of cells. Unattached cells were removed by washing 3 times with warm PBS. The remaining cells were fixed in 3.7% paraformaldehyde and stained with 0.1% toluidine blue in 1% Na Borate. The plates were washed gently with water and the absorbance measured at 570 nM in an ELISA plate reader. In some experiments the cells were photographed using Kodak technical pan film (ESTAR AH base) on an Leica DM IL inverted microscope. The data was graphed using SigmaPlot 5.0.

3.6 Colony Assay

Tissue culture dishes (60 x 15 mm) were coated with 1.5 ml of a 1:1 solution of 2X

RPMI 1640 and 1.2% agar resulting in a final concentration of 1X RPMI 1640 and 0.6% Agar. Once solidified, this layer was overlayed with a 1:1 mixture of 2X RPMI 1640 and 0.72% agar resulting in final concentration of 1X RPMI 1640 and 0.36% agar. In some experiments ECM proteins were added, to the mixture before solidification. The cells were also added to the mixture before solidification but after the solution was below 37°C. The top layer was a total of 2.5 ml and contained 1% penicillin/streptomycin. The colonies were incubated at 37°C for 10-14 days. Once the colonies were clearly visible they were fixed by adding 7 ml methanol to the plates for 30-45 min with occasional stirring. The methanol was then discarded and the plates allowed to air dry for several minutes. PBS containing Geimsa (25:1) was added to the plates and allowed to stain for 45-60 min. The stain was then discarded and the plates were rinsed with a 5 min wash using PBS. If the colonies were not counted immediately the plates were stored at 4°C. The colonies were counted manually by placing the dish over a light box. All visible colonies were counted. The data was graphed using SigmaPlot 5.0.

3.7 Surface labelling with iodine and Biotin for integrin analysis

a) Iodine

Cells were detached from tissue culture flasks with 5 mM EDTA in PBS, washed twice in PBS, the number of cells normalized in each group, and surface labelled with 0.05 mCi ^{125}I in small glass vials in the presence of Iodogen (Pierce) for 30 min at room temperature. The cells were then washed 3 times in PBS to remove free label and lysed in 500 μl Radioimmunoprecipitation assay (RIPA) lysis buffer containing 2.0 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min on ice with agitation. RIPA lysis buffer is a strong detergent which contains 3 different detergents to maximize the solubility of

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proteins embedded in the plasma membrane. The lysate was centrifuged in a microcentrifuge (12,000 rpm) for 10 min and the pellet discarded. The number of cpm in the supernatant was determined by counting a 5 μl aliquot in a Beckman Gamma 7000 gamma counter. The lysate was then aliquoted such that $6x10^6$ cpm were used in each reaction.

b) Biotin labeling

Adherent cells were washed with PBS and harvested using 5 mM EDTA. The cell numbers were normalized in each group and incubated with a final concentration of 100 μg/ml D-Biotin-N-hydroxy succinimide ester (ICN) (reconstitued in DMSO) in RPMI 1640 medium for 40 min at 4°C. The cells were then washed 3 times with PBS and harvested in PBS. The cells were counted and the cell number normalized between cell lines. The cells were lysed in RIPA lysis buffer containing 2.0 mM PMSF directly on the tissue culture plate. Immunoprecipitations were conducted as described in section 3.8 after normalizing the protein concentration in each group.

3.8 Immunoprecipitation for analysis of integrin

Anti-integrin antibodies were added to the lysate aliquots and incubated overnight at 4°C on a tube rotator. The primary antibodies of mouse origin required the addition of a rabbit anti-mouse secondary antibody which was added several hours after the primary antibody. The following day, 50 μl protein A Sepharose in PBS was added to the lysate and incubated at 4°C on a tube rotator for at least 2 h. The immunoprecipitates were washed twice with RIPA containing 0.5 M NaCl and twice with RIPA (no NaCl) both containing 1.0 mM PMSF. The protein A Sepharose beads containing the antibody-integrin complexes were resuspended in 60 μl Laemmli sample buffer (62.5mM Tris, 10% glycerol, 2.3% SDS

and bromophenol blue) and boiled for 3 min. The samples were then resolved on a 7.5%
SDS-polyacrylamide gel under nonreducing conditions. Once the dye front had migrated
off the gel, the gel was fixed in gel fixative (10% glacial acetic acid, 37.5% methanol in
dH_2O, and bromophenol blue) for at least 3 h with agitation. The gel was then rinsed in gel
fixative without bromophenol blue for several minutes, then rinsed with dH_2O and placed
on Whatman filter backing paper and covered with saran wrap. The gel was then dried in
a Bio-Rad model 583 Gel Dryer for 1.5 h at 80°C and subjected to autoradiography using
Kodak Diagnostic X-OMAT AR Film.

3.9 Immunoprecipitation of HGF receptor, FAK and PI 3-kinase

Cells were grown to sub-confluency in large 150 x 20 mm tissue culture plates and
serum-starved for 24 h. The cells were harvested using 5.0 mM EDTA and pooled in one
tube in RPMI 1640 supplemented with 0.5 mg/ml BSA. The cells were then resuspended
in RPMI 1640 supplemented with 0.5 mg/ml BSA and equal volumes aliquoted in each
group. The adherent cells were incubated in the incubator at 37°C while the non-adherent
groups were kept in 10 ml tubes in a 37°C waterbath. After 1h (30 min for HGF studies
alone), the cells were rinsed with cold PBS buffer and lysed in a buffer containing 50 mM
Tris (pH 7.5), 150 mM NaCl, and 1% NP-40, 1 mM sodium orthovanadate, 50 mM NaF,
2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF. A strong detergent such as RIPA is not
required for solubilization of cytoplasmic proteins. In addition the milder detergent will
help preserve protein-protein interactions so that co-precipitation studies can be performed.
The lysates were incubated on a rotator at 4°C for 30 min then centrifuged for 10 min at
14,000 rpm in an IEC/Micromax centrifuge at 4°C. The lysates were precleared with rabbit
anti-mouse IgG and protein A sepharose. After preclearing with rabbit anti-mouse IgG and

protein A sepharose a 50 μl aliquot was removed and the protein content determined using the BCA protein assay kit. The lysates were then incubated with the indicated antibodies at 4°C for 1 h or overnight. Immunoprecipitates were collected on protein-A sepharose, washed three times with lysis buffer, separated on 8.0 or 10% SDS-PAGE under reducing conditions (5% 2-mercaptoethanol).

3.10 Cell Survival assay

Corning non-tissue culture dishes were coated with a 1:1 mixture of 1.2% agar/2X RPMI 1640 and allowed to solidify. The mixture was added to the plate and removed immediately, leaving only a thin layer. SP1 cells were harvested and washed once in RPMI 1640 supplemented with 0.5 mg/ml BSA. Cells were added (20,000/dish) in a total of 1.5 ml volume. In some groups FN, Coll I or LM was added at a concentration of 10 μg/ml or HGF at 5 μg/ml unless indicated otherwise. The plates were incubated at 37°C for 24 h. The cells were harvested and stained with 4 μg/ml acridine orange and 4 μg/ml ethidium bromide then examined under fluorescence using an I filter. The nuclei of viable cells will stain green with acridine orange and cytoplasmic RNA will stain red/orange with ethidium bromide. The nuclei of non-viable cells will stain red/orange nuclei with the ethidium bromide. At least 100 cells were counted and scored as dead or alive based on the nuclear staining. To avoid bias in the results the experiment was prepared by one individual and counted by another. The groups were given number or letter designations so that the person counting was unaware of the condition in each group. The data was graphed using SigmaPlot 5.0.

3.11 Western Blotting

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After the proteins were run on SDS-PAGE they were transferred to nitrocellulose in a Biorad transfer apparatus using Towbin transfer buffer (25 mM TRIS, 192 mM Glycine, 20% Methanol). The nitrocellulose was then dried at 4°C and blocked with 5.0% skim milk powder in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 1h. The blot was then washed 3 times (10 min each) and incubated with the primary antibody (1:1000) at room temperature for 1 h. The blot was washed again 3 times with TBST and incubated for 1 h at room temp with the horse-radish peroxidase secondary antibody (1:5000). The blot was then washed 4 times with TBST and treated with ECL reagents and developed on Dupont reflection film. The films were then scanned on a Hewlett Packard (HP) ScanJet 3c and saved as a tif file. The file was then imported into Corel Draw 3.0 and the labels added to the figure. The finished figure was printed on an HP LaserJet 5MP at 600 DPI.

3.12 Cell proliferation assay:

SP1 cells were seeded (20,000/well) in 24 well plates under the various conditions indicated in 1 ml of medium. DNA synthesis was measured at 48 h by adding 0.2 μCi ^{3}H-thymidine at 24 h. After an additional 24 h, cells were harvested with trypsin/EDTA. Aliquots of cells were placed in 96-well microtitre plates and transferred to filters using a Titertek cell harvester (Flow Laboratories), and ^{3}H-thymidine incorporation was measured in a scintillation counter (Beckman).

3.13 PI 3-kinase Assay:

The cells were prepared and PI 3-kinase was immunoprecipitated from SP1 cell lysates with anti-PY IgG, as described above. PI 3-kinase activity was measured by incubating

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immunoprecipitates with PI (20 μg), 100 mM $MgCl_2$, 0.88 mM ATP, [γ-^{32}P]-ATP (30 μCi) and a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, for 20 min with constant agitation at room temperature. Reactions were terminated by the addition of 20 μl of 6 N HCl and lipids were extracted by the addition of 160 μl of $CHCl_3$:CH_3OH (1:1) and were separated in CH_3Cl_3:CH_3OH:H_2O: NH_4OH (60:47:11.3:2) by thin layer chromatography (TLC). The TLC plate was dried and autoradiographed, and the radio labeled PI 3-P spots were scraped from the plate and counted by liquid scintillation.

3.14 Cell Transfection:

The cDNA encoding the mutant bovine p85 (Δp85) subunit of PI 3-kinase which lacks a binding site for the p110 catalytic subunit of PI 3-kinase was ligated into the SRα plasmid as described (Hara et al., 1994). Expression of this mutant results in the deletion of 35 amino acids from residues 475-513 of p85α and contains an insertion of two other amino acids (Ser-Arg) at this deleted position. SP1 cells expressing the mutant Δp85 were established using the stable transfection lipofectamine method according to the manufacturer's instructions. The transfections were performed and selected by the technician in our laboratory. Briefly, SP1 cells were grown to 80% confluency. The DNA (1 μg) mixed with lipofectamine reagent (1 μl) in 200 μl serum-free medium and incubated for 15 min at room temperature. Before transfection, cells were washed once with 2 ml of serum-free medium. For each transfection, the mixed DNA and lipofectamine is combined with 0.8 ml of serum-free RPMI medium and the cells were incubated with this solution. After 5 hr of incubation, one ml of 14% FBS was added, without removing the transfection medium for additional 24 hr. Subsequently, twenty-four clones were selected in G418 (250 μg/ml) and expression of Δp85 was assessed by western blot analysis with anti-bovine p85

IgG.

Statistical analysis

There are four types of assays presented in this thesis as bar or line graphs:(1) adhesion assays,(2) colony assays, (3) growth assays, and (4) survival assays. For adhesion assay and colony assays we have chosen to present one representative experiment as opposed to the mean of several experiments. The data in these assays show a lot of variability between experiments and although treatment groups respond similarly compared to controls, a large number of experiments would be required to obtain statistical significance in each case. The groups were performed in triplicate and I have stated the number of times the experiment was performed in the figure legend and/or text. Another method of presenting these data would be to present the data from several experiments combined into one figure. We did not present the data in this form since it would complicate the figure considerably and not all treatment groups were done in all experiments due to reagent availability and cost.

We have used ANOVA on the growth assay and all survival assays using the MGLH module of the Systat package for DOS. The null hypothesis that all groups were equal was tested at the 0.05 level of significance. We then performed a post hoc analysis of pairwise comparisons using the Bonferoni adjustment for multiple comparisons.

CHAPTER 4

EXTRACELLULAR MATRIX - INTEGRIN INTERACTIONS IN SP1 CELLS
UNDER ADHERENT AND ANCHORAGE-INDEPENDENT CONDITIONS

4.1 Rationale and Objectives

Adhesion to the extracellular matrix can affect many cellular properties including cell shape, growth, migration, survival, differentiation, invasion and gene expression (von der Mark et al., 1992). The two primary mechanisms by which cells alter adhesion to the extracellular matrix are (1) through changes in the expression of cell surface receptors and (2) through changes in secretion of ECM proteins. Expression and distribution of ECM proteins is a dynamic process especially during development and pathogenesis. For example, changes in ECM expression occur during normal development of the breast tissue, during lactation and involution (Howlett and Bissell, 1993). In contrast, an increase in proteases and ECM proteolysis precedes apoptosis of mammary cells during involution, hence the ECM acts as a survival factor (Pullan et al., 1996).

Integrins also play a key role in both normal and tumor cell function. Transformed cells are generally less adherent than their normal cell counterparts but maintain a broad expression of integrins. It is not known whether a disorganized pattern of integrin expression in carcinomas is caused by the disruption of the ECM or whether the reverse is true, i.e. changes in integrin expression affect ECM expression. The search for predictable patterns of integrins which correlate with the transformed phenotype has been confusing and unsuccessful. There are, however, a few emerging trends in the expression of integrins during malignancy. For example, a decrease in the fibronectin receptor, $\alpha_5\beta_1$, and an increase in the laminin receptor, $\alpha_6\beta_1$, are common in more malignant epithelial

tumors (Tang and Honn, 1995). An increase in $\alpha_v\beta_3$ expression is common in malignant melanomas and correlates with the invasive phenotype (Seftor et al., 1992). Similar examples are common but such increases or decreases do not occur in all transformed cells. In addition, other integrins do not appear to follow any predictable patterns of expression during transformation, making the role of such receptors difficult to assess.

Growth factors and cytokines also affect ECM interactions with both normal and transformed cells. Numerous reports have shown that growth factors such as PDGF, EGF, FGF, HGF and TGF-β can affect cell adhesion, spreading and migration and can increase anchorage-independent growth of a number of cell lines (Rizzino et al., 1986; Newman et al., 1986; Kanda et al., 1993). Rizzino et al (1986) also showed that TGF-β could modulate colony growth of NRK cells depending on which other growth factors were present.

Both primary and metastatic solid tumors tend to grow in spheroid-like structures in vivo. These spheroids contain extracellular matrix proteins such as fibronectin, collagen, laminin and proteoglycans (Glimelius et al., 1988). However, their function in tumor spheroids is not known. In a more defined system, a greater proportion of mammary cells will maintain β-casein expression in a three-dimensional matrix as compared to cells cultured on plastic (Roskelley et al., 1994). The mechanisms which regulate these differences are not known but it is suggested that cell shape is an important factor in determining the response to ECM (Watt, 1986). These responses activate a number of intracellular signaling pathways. For example, Roskelley and Bissell (1995) have shown that the tyrosine kinase inhibitors genistein and staurospaurine inhibited milk production in mammary cells maintained on the basement membrane matrix, Matrigel. In

conclusion, ECM, integrins and growth factor expression play a crucial role in modulating growth and differentiation of both normal and transformed mammary cells (Petersen *et al.*, 1992).

We have previously investigated the presence of ECM proteins in SP1 colonies by immunofluorescence (Appendix I). Fibronectin is the most abundant ECM protein and was present in the form of fibrils. Vitronectin and LM were also present but in lesser amounts. Collagen type IV and tenascin were not detected in SP1 colonies. Collagen type I distribution in SP1 colonies remains to be examined. These observations suggest a role for FN in colony growth. In addition, we have shown that HGF is strongly mitogenic for SP1 cells (Rahimi *et al.*, 1994). However, the role of ECM proteins and more specifically FN on anchorage-independent growth of mammary cells is not well understood. The adhesive and integrin profiles of the murine mammary carcinoma cell line, SP1 have not been previously investigated, thus we must first carefully determine the adhesive and integrin profile of SP1 cells before determining what role ECM-integrin interactions will have on anchorage-independent growth.

In this chapter, our specific objectives are:

1. To establish the adhesive and integrin profiles of SP1 cells.

2. To determine the primary integrin receptors for fibronectin.

3. To determine whether growth factors such as HGF and TGF-β can affect SP1 cell adhesion to ECM proteins.

4. To determine the role of ECM proteins (fibronectin and collagen type I) and growth factors (HGF and TGF-β) in SP1 colony growth.

RESULTS

4.2 SP1 cells adhere to fibronectin and express the integrin $\alpha_5\beta_1$

SP1 cells are an adherent murine mammary carcinoma cell line. They adhered and spread extensively to fibronectin and vitronectin over a wide range of concentrations (0.16-20 μg/ml). They adhered moderately well to collagen type I, and show a concentration dependent requirement of approximately 1.5 μg/ml to collagen type IV. SP1 cells attached poorly to laminin and few cells remained on BSA coated plates after washing with PBS (Figure 4). Although SP1 cells adhere poorly to LM, we show that at concentrations greater than 2.5 μg/ml we see more SP1 cells adhering to LM as compared to the BSA control (Figure 4). We observed a positive correlation between the degree of spreading and the strength of adhesion of SP1 cells. SP1 cells adherent on FN and VN show a more flattened morphology as compared to Coll I and LM, onto which they spread poorly and show a spindle-shaped morphology (Figure 5).

Cell adhesion to the ECM can regulate integrin expression (Chen *et al.*, 1992; Dalton *et al.*, 1992), therefore we determined the integrin profiles of SP1 cells cultured on plastic or anchorage-independently for 24 h in the presence of 7% FBS. In Figure 6 we have used a number of different antibodies to immunoprecipitate ^{125}I-labelled integrins from the cell surface of SP1 cells. Integrin α subunits are typically 120-150 kDa, with the exception of α_1 subunit which is 180 kDa. The β_1 subunit is 110 kDa and the β_3 subunit is 97 kDa. Immunoprecipitations using antibodies directed to one subunit will often co-precipitate the associated subunit(s). For example in the anti-β_1 immunoprecipitation in Figure 6, the antibody is directed to the β_1 subunit and will co-precipitate all associated α subunits. Therefore the upper band, which is difficult to distinguish from the lower band

in this exposure, is composed of numerous α subunits, and the lower band of the β_1 subunit. In the immunoprecipitation using antibodies specific for α_2, α_3, α_5, α_6, or α_v subunits, co-precipitation of the corresponding β subunit is also observed. The association between some α and β subunits is not strong, and may be lost when using a strong lysis buffer such as RIPA. For example, there is only a weak β band associated with the α_v, α_2 and α_6 subunit. A weaker detergent such as NP40 or Triton X-100 can be used to preserve the association between the α and β subunits.

We have precleared the cell lysates with rabbit anti-mouse IgG before immunoprecipitation with the anti-integrin antibodies to help reduce non-specific binding to the IgG. Figure 6 illustrates that the integrin expression under adherent and anchorage-independent conditions is very similar. SP1 cells express a number of integrins on the cell surface including the FN receptor, $\alpha_5\beta_1$, the VN receptor, $\alpha_v\beta_3$, the LM receptor, $\alpha_6\beta_1$ and the CoII, LM and FN receptor, $\alpha_3\beta_1$. The collagen/LM receptor, $\alpha_2\beta_1$, was weakly expressed on SP1 cells cultured on plastic but was upregulated on SP1 cells which were maintained under anchorage-independent conditions for 24 h (Figure 6). A similar gel was repeated 4 times with SP1 cells under adherent conditions or with a metastatic variant of SP1 cells. In the first two integrin profiles performed using ^{125}I-labelled cells, an IgG control was added and no non-specific bands were observed. Flow cytometry revealed very low expression of the FN receptor $\alpha_4\beta_1$ (data not shown). These data show that SP1 cells adhere to a number of ECM proteins and express a number of different integrins on the cell surface.

Integrin receptors require divalent cations such as Ca^{2+}, Mg^{2+}, or Mn^{2+} for adhesion. To provide further evidence that integrin receptors are the primary receptors

for extracellular matrix proteins on SP1 cells we investigated the role of divalent cations on SP1 cell adhesion to fibronectin. We removed the divalent cations from the cell surface by incubating the cells in divalent cation-free PBS containing 5 mM EDTA then resuspending them in divalent cation-free PBS supplemented with 0.5 mg/ml BSA.

In the absence of divalent cations, SP1 cells did not adhere to FN. Addition of Ca^{2+}, and Mn^{2+} at low concentrations (0.1-1.0 mM) promoted adhesion of SP1 cells to FN, while concentrations greater than 1.0 mM were inhibitory and formed a precipitate. Mg^{2+} did not form a precipitate and was effective at promoting SP1 cell adhesion to FN over a wide range of concentrations (0.1-50 mM) (Figure 7). Other divalent cations such as Zn^{2+} or Cu^{2+} did not support SP1 cell adhesion to fibronectin (data not shown).

In conclusion, the results in this section show that SP1 cells can adhere to several ECM proteins and express a number of integrin receptors which require divalent cations to mediate cell adhesion to fibronectin.

Figure 4. <u>Adhesion of SP1 cells to purified ECM proteins</u>

Linbro 96-well tissue culture plates were coated for 18 h at 4°C with BSA, FN, LM, VN, Coll I and Coll IV at concentrations ranging from 0.15 to 20 μg/ml (obtained by a two-fold serial dilution). The wells were washed with PBS and blocked with RPMI supplemented with 0.5 mg/ml BSA. SP1 cells in RPMI 1640 supplemented with 0.5 mg/ml BSA were added to each well (30,000 cells /well) in a 100 μl volume. After 45 min at 37°C, the unattached cells were removed by washing with warm PBS. The remaining cells were fixed with 3.7% paraformaldehyde and stained with a solution of 1% toluidine blue in a 1% solution of sodium borate. The absorbance was measured with an ELISA plate reader at a wavelength of 570 nm. Each point is expressed as the mean $\pm$ SD of triplicates. The experiment was repeated three times with similar results. The symbol designating each ECM protein is indicated in the figure legend at the top left hand corner of the graph.

Figure 5. <u>Morphology of SP1 cells on ECM proteins</u>

SP1 cells were harvested and washed once in RPMI 1640 supplemented with 0.5 mg/ml BSA. They were then seeded (50,000 cells/well) in Costar 24-well tissue culture plates (coated with the indicated ECM proteins at 10 μg/ml) and allowed to adhere. Nonadherent cells were not removed. After 45 min the cells were photographed using a Leica DM IL inverted microscope using technical pan film (ESTAR-AH-base). Magnification: 66X

SP1 cells on (A) Fibronectin

(B) Vitronectin

(C) Collagen type I

(D) Laminin
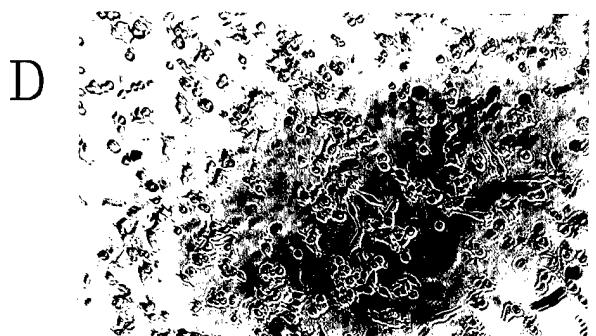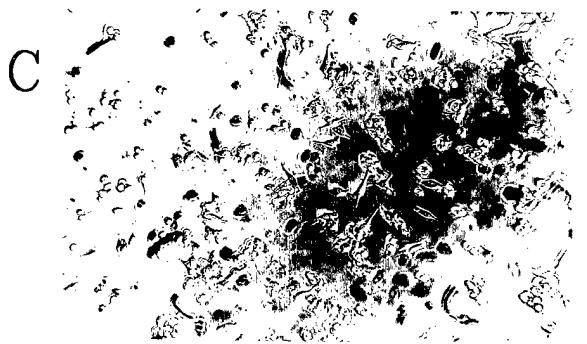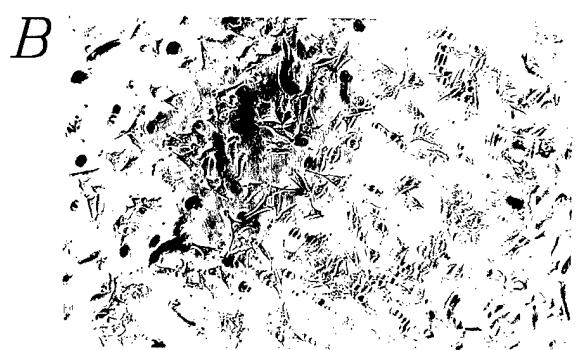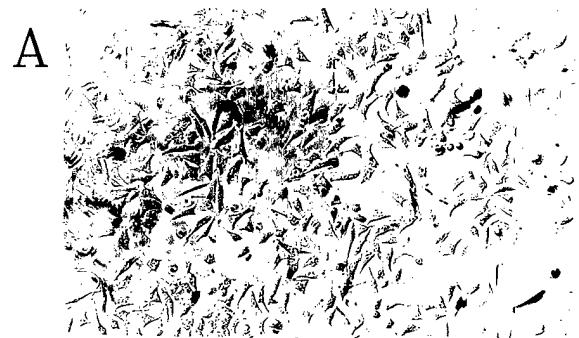
Figure 6. Analysis of SP1 integrin profile cultured on plastic and agar.

SP1 cells were cultured on tissue culture plastic or as colonies on agar coated plates in RPMI 1640 medium supplemented with 7% FBS for 24 h. The cells were harvested and the number of cells normalized in each group, then surface labelled with 1.0 mCi ^{125}I . The cell pellets were lysed in RIPA buffer at 4°C for 30 min and the supernatants were precleared with rabbit anti-mouse IgG and protein A sepharose. The supernatents were aliquoted and incubated with anti-integrin antibodies and protein A sepharose. Immunoprecipitates were washed 4 times in lysis buffer and run on a 7.5% SDS-PAGE under non-reducing conditions. Antibodies used were; from left to right, polyclonal anti-β_1; polyclonal anti-human-α_2 (cytoplasmic); polyclonal anti-human-α_3 (cytoplasmic); polyclonal anti-human-α_5 (cytoplasmic); mouse monoclonal anti-α_6 (GoH3) and polyclonal anti-human-α_v (cytoplasmic). The gel was fixed in gel fixative (10% acetic acid, 40% methanol, 50% dH$_2$O) and dried at 80°C for 1.5 h and autoradiographed. A similar gel was repeated 4 times with similar results.

Legend:

"P"- cells grown on tissue culture plastic (adherent)

"A" - cells grown on agar (non-adherent)

NOTE: cytoplasmic indicates that the antibody is directed to the cytoplasmic domain of the integrin subunit.

Kda	β_1		α_2		α_3		α_5		α_6		α_v	
	P	A	P	A	P	A	P	A	P	A	P	A
206--												
105--												
70--												

Figure 7. <u>Effect of divalent cations on SP1 cell adhesion</u>

SP1 cells were harvested with 5 mM EDTA and washed once in PBS supplemented with 0.5 mg/ml BSA. The cells were resuspended in divalent cation-free PBS supplemented with 0.5 mg/ml BSA and seeded (30,000 cells/well) in Linbro 96-well tissue culture plates coated with BSA or FN (10 μg/ml) as described in the Materials and Methods. The medium was supplemented with Ca^{2+}, Mg^{2+}, or Mn^{2+} at the indicated concentrations. After 45 minutes, the non-adherent cells were removed by washing 4 times with warm PBS. The remaining cells were fixed with 3.7% paraformaldehyde and stained with 1% toluidine blue in 1% sodium borate. The absorbance was measured using an ELISA plate reader at 570 nm. The results are expressed as the mean $\pm$ SD of quadruplicates. The experiment was repeated twice with similar results.

4.3 BMA5 antibody inhibits SP1 cell adhesion to fibronectin

SP1 cells express several integrin receptors with potential to bind fibronectin ($\alpha_5\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$). These integrins bind to their ligand via the RGD sequence. We therefore tested a GRGDSPK peptide and a control peptide GRGESPK to inhibit adhesion to FN.

In our system, the GRGDSPK peptide was unable to reproducibly inhibit adhesion to fibronectin even at concentrations up to 1.0 mg/ml. However it was very efficient at inhibiting adhesion to vitronectin (data not shown). Blocking antibodies to many murine integrins are not available commercially, however, we obtained a blocking antibody to the murine $\alpha_5\beta_1$ integrin (gift from B. Chan). Preliminary experiments using FACS analysis with BMA5 as hybridoma conditioned medium and affinity purified IgG demonstrated that the antibody reached binding saturation to SP1 cells at 25-30% v/v for the hybridoma conditioned medium and at 5 μg/ml for the affinity purified BMA5 antibody (data not shown). Based on these results we used the BMA5 (hybridoma conditioned medium) at a concentration of 30% v/v and in affinity purified form at 10 μg/ml. BMA5 (hybridoma conditioned medium) was effective at inhibiting adhesion of SP1 cells to FN while the antibody in affinity purified form was also able to inhibit adhesion but was less efficient (Figure 8). The decrease in efficiency is likely a result of denaturation resulting from the purification process. A rabbit anti-mouse IgG control or the blocking antibody GoH3 (hybridoma conditioned medium) which recognizes the laminin binding integrin, $\alpha_6\beta_1$, did not inhibit adhesion to FN. The GoH3 antibody was used as a control for the hybridoma conditioned medium (Figure 8).

The results in this section demonstrate that $\alpha_5\beta_1$ is the primary integrin receptor for FN on SP1 cells.

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Figure 8. <u>Adhesion assay in the presence of BMA5 antibody</u>

Panel A

Linbro 96 well tissue culture plates were precoated with fibronectin (10 μg/ml) or

BSA (10μg/ml) overnight at 4°C. The wells were washed with PBS and blocked with

RPMI 1640 supplemented with 0.5 mg/ml BSA for 2 h at 37°C. SP1 cells were seeded

(30,000 cells/well) in the presence of no antibody (NT), GoH3 (hybridoma conditioned

medium) (30% v/v), BMA5 antibody (hybridoma conditioned medium, 30% v/v) (BMA5

(sup)) and affinity purified BMA5 (10 μg/ml) (BMA5(conc)), or rabbit anti-mouse IgG (30

μg/ml) (IgG), After 30 min at 37°C the nonadherent cells were removed by washing with

warm PBS. The remaining cells were fixed in 3.7% paraformaldehyde. The cells were

stained in 1% toluidine blue and the absorbance measured in an ELISA plate reader at 570

nm. The results are expressed as the mean $\pm$ SD of triplicates. The experiment was

repeated twice with similar results.

4.4 TGF-β increases adhesion of SP1 cells to the ECM

Transforming growth factor-β (TGF-β) is well recognized in the literature for its ability to increase both extracellular matrix secretion and integrin expression (Heino *et al.*, 1989; Roberts *et al.*, 1992). We investigated whether TGF-β could promote adhesion of SP1 cells. SP1 cells were treated with TGF-β in the presence of 2% FBS under both adherent and anchorage-independent conditions. The serum concentration was reduced to 2% to minimize the effects of the serum but maintain viability of the cells in suspension cultures. When cells were treated with TGF-β under adherent conditions there was an increase in adhesiveness to FN, VN and Coll I and IV as determined by an adhesion assay (Figure 9A). SP1 cells did not adhere significantly to laminin with or without TGF-β treatment. When SP1 cells were treated with TGF-β under anchorage-independent conditions there was an increase in adhesiveness to Coll I and IV but the adhesiveness to FN, VN or LM remained unchanged (Figure 9B). In both cases the increase in adhesiveness to Coll I was greater than that to Coll IV. We are currently investigating the ability of TGF-β to alter ECM expression by Northern analysis and integrin expression by cell-surface labelling in SP1 cells under adherent and anchorage-independent conditions.

We also investigated whether HGF could alter the adhesive potential of SP1 cells. Our results showed that HGF does not alter the adhesive potential of SP1 cells on FN or VN but does stimulate cell spreading (data not shown). In addition, others have reported that HGF does not alter integrin expression (Matsumoto *et al.*, 1994).

The results in this section show that TGF-β but not HGF can alter the adhesive potential of SP1 cells to extracellular matrix under both adhesive and anchorage-independent conditions.

Figure 9. <u>Effect of TGF-β on SP1 cell adhesion.</u>

SP1 cells were cultured under (A) adherent or (B) anchorage-independent conditions (in agar coated plates) in the presence of 2% FBS and in the absence or presence of 0.5 ng/ml TGF-β for 24 h. SP1 cells were harvested and seeded (30,000/well) in 96-well plates precoated with BSA, FN, VN, LM, Coll I, and Coll IV at a concentarion of 10 μg/ml as described in the Material and Methods. After 45 min at 37°C, the non adherent cells were removed by washing with warm PBS and the remaining cells were fixed and stained as previously described. The results are expressed as mean ± SD of triplicates. Panel A: Adhesion assay of SP1 cells treated with TGF-β under adherent conditions

Panel B: Adhesion assay of SP1 cells treated with TGF-β under anchorage-independent conditions.

A

B

4.5 Exogenous fibronectin is required for adequate colony formation

We have just shown that SP1 cells adhere well to FN via the $\alpha_5\beta_1$ integrin and we have previously demonstrated that FN is abundant in SP1 colonies (Appendix I). Thus, the next step in investigating the role of extracellular matrix proteins, especially FN, in anchorage-independent growth was to remove the primary source of FN from the colonies. Fibronectin was partially removed from FBS by immunoprecipitation with anti-bovine FN antibodies (Telios). Partial removal of FN from serum reduced colony growth efficiency of SP1 cells by approximately 50% whereas endogenous IgG depletion with protein A Sepharose alone or preclearing of the serum with rabbit anti-mouse IgG did not remove any factors required for growth in soft agar (Figure 10). Addition of exogenous bovine plasma FN at 10 μg/ml to the FN reduced-serum partially reconstituted the colony growth. We have observed a reduction in colony growth in 4 of 6 experiments with a range of 26-89% inhibition. We have also analysed the immunoprecipitates of FN from serum on 6.0% SDS-PAGE under reduced conditions. Silver staining the gel demonstrated that we were able to remove some FN from the serum (data not shown). A positive control of 10 μg of purified bovine plasma FN in an adjacent lane. Although FN was likely not completely removed from the serum, our results show that we have removed sufficient FN from the serum to affect colony growth. Further experiments are required to determine the extent of the FN removed from the serum in our system by western blot analysis of the serum with FN removed by immunoprecipitation. It is possible that further depletion of FN will result in a greater and more consistant reduction in colony growth.

Another common method of removing FN is using gelatin sepharose beads to which FN has a strong affinity. In our system, this method is not practical since it requires

dilution of the solution containing FN in a buffer which would affect the serum and inhibit use in tissue culture. Since removal of FN from serum was incomplete, we attempted another approach by adding FN to low serum conditions.

We next investigated whether addition of purified bovine plasma FN as well as other ECM proteins to limiting culture conditions (1% FBS) would enhance colony growth. When SP1 cells are cultured in agar containing 1% FBS, very few colonies grow and remain small in size as compared to those obtained in the presence of 7% FBS. In the complete absence of serum, SP1 cells do not form any colonies in soft agar. We used these limiting conditions (1% FBS) to determine which ECM proteins affect anchorage-independent growth of SP1 cells. The addition of FN and LM was shown to increase colony growth marginally in 1% FBS, but likely lacked the needed mitogenic components present in 7% FBS to generate large colonies (Figure 11). The increase in colony growth observed with FN, although small, was reproducible in 4 of 4 experiments and the range of increase was from 29-63% increase compared to 1% FBS control. In contrast, both Coll I and IV inhibited colony growth of SP1 cells with the former being more effective. Collagen type I could inhibit colony growth in the presence of both 1% or 7% FBS (Figure 11, inset). Addition of Coll I or IV with FN or LM, respectively, also causes a reduction in colony growth demonstrating a dominant effect of Coll. We have observed a decrease in colony formation in the presence of Coll I in 4 of 7 experiments. In the 4 experiments where a decrease was observed, the range was from 46-96%.

We have previously shown that FN in SP1 colonies is present in the form of fibrils (Appendix I). We now show that the 70 amino-terminus kDa fragment of FN (see Figure 1), which inhibits FN fibril formation by causing chain termination, reduces colony growth

of SP1 cells. We have shown that addition of the 70 kDa fragment reduces colony growth by approximately 30% at a concentration of 10 μg/ml in agar as compared to a control 85 kDa peptide which does not inhibit FN fibril formation (Table 2). The 70 kDa fragment of FN does not inhibit SP1 cell adhesion to FN therefore its effect on colony growth is mediated through inhibition of fibril formation and not through interference of FN-integrin binding. We also incubated SP1 cells under anchorage-independent conditions with FITC-labelled FN for 5 days. These results show that exogenous FITC-labelled FN at 30 μg/ml was incorporated in SP1 colonies especially at the early stages, since FITC-labelled FN appears to be primarily in the central region of the colony (Figure 12).

The results in this section show that an exogenous source of FN and assembly into fibrils is required for adequate colony formation of SP1 cells and that collagen inhibits SP1 colony formation.

Figure 10. <u>Effect of FN depletion on SP1 colony growth</u>

Serum was either untreated (UT) or incubated with protein A sepharose (-PAS) to remove endogenous IgG. After PAS treatment the serum was precleared with rabbit anti-mouse IgG (-IgG) and PAS. After preclearing the serum, fibronectin was removed by immunoprecipitation with a polyclonal anti-FN antibody ($5\mu l$/ml serum)(-FN). In one group bovine plasma fibronectin was added to the culture medium at a concentration of 10 μg/ml (-FN+FN). The colony assay was performed as described in the Materials and Methods in the presence of 7% FBS. The cells were incubated for 14 days then fixed in methanol and stained with Geimsa. The colonies were counted manually. The results are expressed as the mean $\pm$ SD of quadruplicates. The experiment was done 6 times and we observed an inhibition in 4 of the experiments (76%, 89%, 75% and 26%).
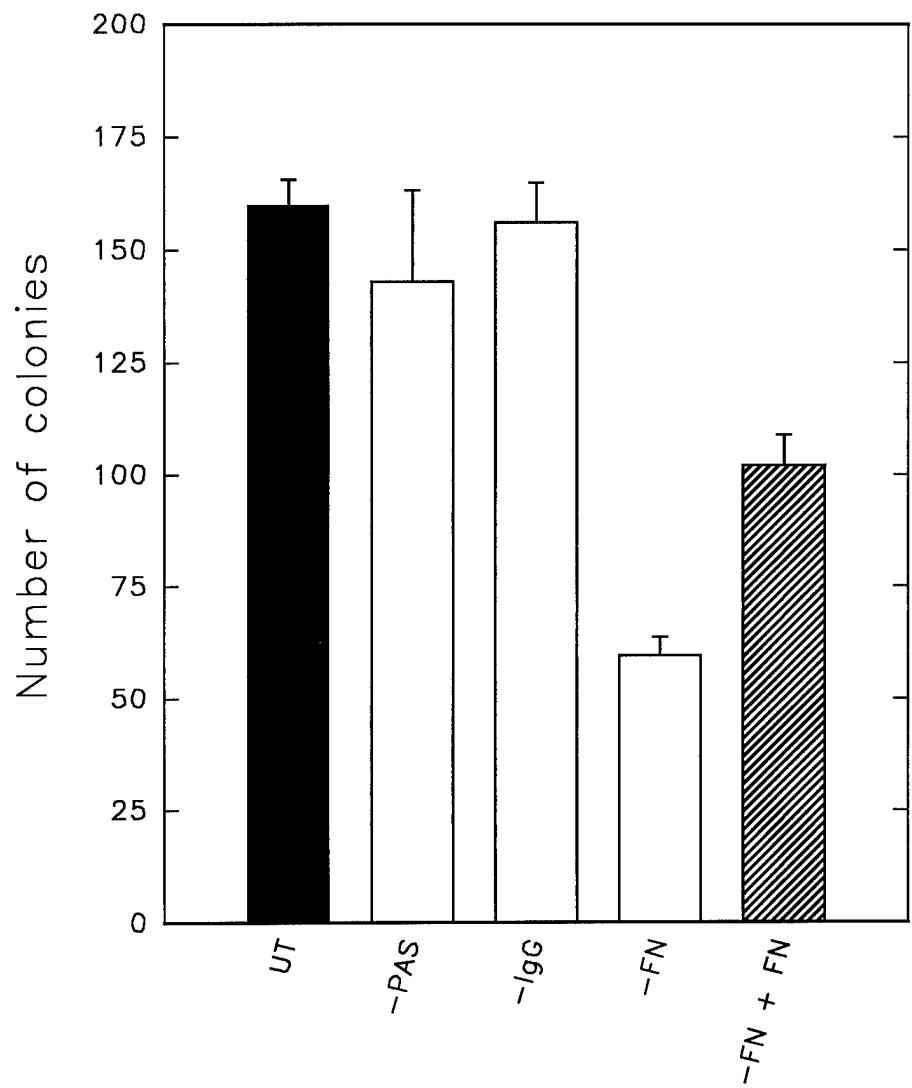
Figure 11. <u>Effect of purified ECM proteins on SP1 colony growth</u>

The colony assay was performed as described in the Material and Methods. The purified matrix proteins indicated in the X axis were added at a concentration of 10 μg/ml to the RPMI 1640/agar mixture before solidification. All cultures contained 1% FBS. <u>Inset:</u> shows the effect of collagen type I on SP1 colonies in medium supplemented with 1% and 7% FBS. Colony growth was allowed to proceed for 14 days at 37°C. The colonies were fixed and stained as described in the Materials and Methods. The results are expressed as the mean ± SD of quadruplicates. The experiment was repeated four times. The colony assay with collagen was performed a total of 7 times. An inhibition of colony growth with Coll I was observed in 4 of the experiments (46%, 83%, 46% and 96%).

Table 2

Effect of the 70 kDa and 85 kDa fragments of fibronectin on SP1 cell colony growth

Fibronectin fragments	Number of colonies
3% FBS	90 ± 6.5
3% FBS + 70 kDa FN Fragment	62 ± 4.1
3% FBS + 85 kDa FN Fragment	91 ± 11

Legend:

SP1 were seeded (30,000/well) in 0.75 ml of a 1:1 mixture of agar and RPMI 1640 medium in 35 x 10 mm dishes. The final concentration of agar was 0.36%. The 70 kDa N-terminal fragment or the 85 kDa fragment were added to the mixture at 10 μg/ml before solidification. The dishes were incubated at 37°C for 10 days. The colonies were then fixed in methanol, stained with Giemsa and counted manually. The results are expressed as the mean ± SD of quadruplicates.

Figure 12. Incorporation of FITC-labelled FN in SP1 colonies

SP1 colonies were grown on RPMI/agar coated plates supplemented with 3% FBS

partially depleted of FN and 30 μg/ml of FITC-conjugated FN. After 5 days, the colonies

were fixed in 3.7% paraformaldehyde, dried on glass slides, and viewed with a Meridian

confocal microscope. (A) Phase contrast illumination; (B) fluorescence illumination.

Magnification 1000X. The control, containing 30 μg/ml unlabelled FN, showed no

immunofluorescence (data not shown).

4.6 TGF-β and HGF promote SP1 colony growth in 1% serum

We have shown that ECM proteins are required but not sufficient to maintain colony growth of SP1 cells. It is likely that growth factors are also required for adequate colony growth. SP1 cells respond to a number of growth factors including bFGF, PDGF, IGF, HGF, and TGF-β. Although most of these factors have been shown to promote anchorage-independent growth of various cell lines, TGF-β is well established in the literature to promote anchorage-independent growth and modulate both ECM and integrin expression (Heino et al., 1987). In addition, SP1 cells express high levels of the HGF receptor, c-Met, and secrete HGF therefore it is an important autocrine mitogenic factor for SP1 cells (Rahimi *et al.*, 1996b).

Thus, we investigated the ability of these two growth factors to promote anchorage-independent growth of SP1 cells. HGF at 5 ng/ml and TGF-β at 0.5 ng/ml both promoted anchorage-independent growth of SP1 cells in soft agar. However, TGF-β was considerably more efficient than HGF or FN in promoting colony growth in 1% FBS. When added in combination with FN, HGF or TGF-β showed a marginal additive effect compared to either growth factor alone (Figure 13). Concentrations of HGF greater than 10 μg/ml had a negative effect on SP1 colony growth (data not shown).

The results in this section show that both HGF and TGF-β can increase colony growth of SP1 cells whereas FN showed only a marginal effect.

Figure 13. <u>Effect of HGF and TGF-β on SP1 colony growth</u>

The colony assay was performed as described in the Materials and Methods. The control group contained 1% FBS only, FN (10 μg/ml), HGF (5 ng/ml) and TGF-β (0.5 ng/ml) were added to the agar before solidification. The colonies were incubated for 14 days at 37°C. The colonies were fixed and stained as previously described. The experiment was performed in quadruplicate. The results are expressed as the mean $\pm$ SD of quadruplicates. The experiment was repeated 2 times with similar results.
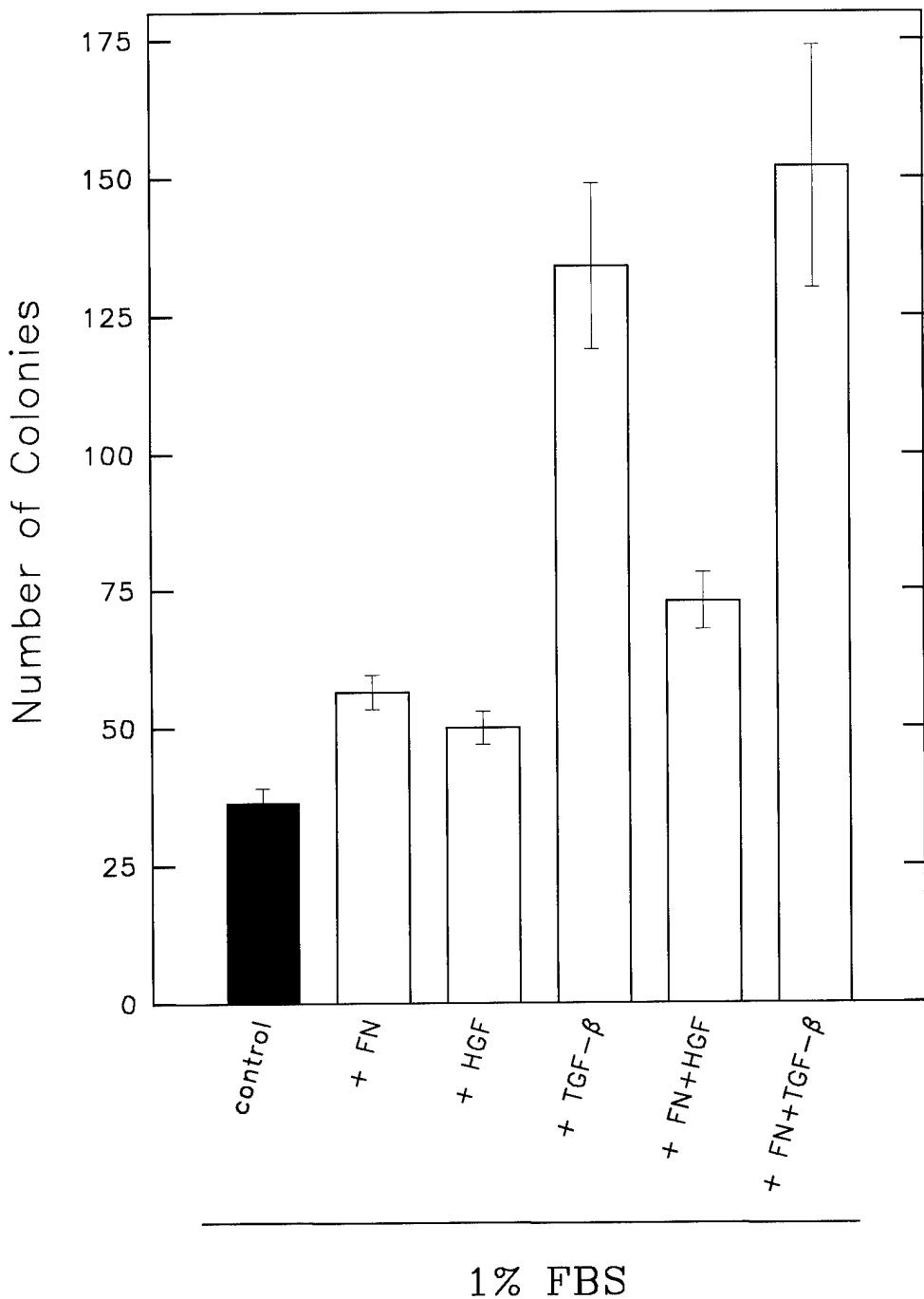
4.7 Discussion

The results in this chapter show that SP1 cells, like many epithelial-derived tumors, adhere to a number of ECM proteins and express several integrin receptors. We have shown that:

1. SP1 cells adhere to fibronectin, vitronectin, collagen type I and IV and express $\alpha_2\beta_1$ $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_v\beta_3$ integrin receptors.

2. SP1 cells adhere to fibronectin primarily via the $\alpha_5\beta_1$ integrin receptor.

3. TGF-β, increases adhesion potential of SP1 cells to fibronectin, vitronectin and collagen when traeted under adherent conditions and only to collagen under anchorage-independent conditions.

4. Fibronectin and fibronectin fibril assembly are required for optimal colony growth.

5. HGF and TGF-β promote anchorage-independent growth of SP1 cells in 1% FBS.

In general, mammary tumor cells often down-regulate integrin expression (Restucci et al., 1995). However, there is conflicting evidence as to the expression of $\alpha_5\beta_1$ in mammary cells. In normal mammary cells $\alpha_5\beta_1$ is not detected or expressed at very low amounts using immunohistochemical techniques. However, $\alpha_5\beta_1$ expression is increased on some transformed cell lines but appears to be down-regulated in other studies (Koukoulis et al., 1991; D'ardenne et al., 1991). Another study showed positive staining in many normal, benign and malignant canine mammary tumors but was unable to show a relationship between $\alpha_5\beta_1$ expression and the degree of malignancy (Peña et al., 1994). Interestingly, $\alpha_5\beta_1$ appears to be increased in malignant, undifferentiated bladder carcinomas as compared to normal and benign cells (Saito et al., 1996). In this chapter

we have shown that SP1 cells adhere well to FN and express $\alpha_5\beta_1$, which is different from mammary cells examined *in vivo*, which reduce the expression of FN after transformation. During this study, several clones of SP1 cells were selected (discussed in chapter 5) and tested for their ability to adhere to FN, VN, LM, Coll types I and IV. We observed that all clones adhered and spread well to FN and VN. In contrast, there was considerable variability in adhesion to LM and Coll types I and IV. This phenomenon is the result of clonal selection in tissue culture. These cells are continually subcultured in medium supplemented with serum. The most prominent adhesive components in serum are FN and VN, whereas only trace amounts of other adhesive proteins are present. During the first few passages after removal of the tumor from the mammary tissue, any cell which does not express receptors for FN or VN would not attach to the tissue culture plastic and would either die or be lost during subsequent tissue culture. SP1 cells are therefore selected for their adhesive potential to serum components.

Most studies of integrin expression in humans or animal tumors have relied upon immunohistochemical techniques. In normal epithelia, integrins that bind Coll, LM, and other basement membrane components tend to be expressed mainly at the basolateral surface, in proximity to the basement membrane. For example $\alpha_2\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ show this type of organization. By contrast, in carcinomas, the spatial arrangement of these integrins becomes quite disordered, with a diffuse and apparently less abundant intracellular distribution. In addition to changes in the spatial pattern of integrin expression, some authors report a reduced level of expression of some integrin subunits in carcinomas, especially α_2, α_3 and α_5. However, as most of these studies relied on immunohistochemistry, it is not clear whether this actually represents a quantitative

difference in the total amount of integrin subunit protein, as opposed to a more diffuse distribution of the same amount of protein (Juliano and Varner, 1993). In addition, these studies are performed on tumor cells with a different microenvionment than those in culture. Dalton *et al* (1992) demonstrated a reduction in $\alpha_5\beta_1$ expression in both transformed and non-transformed fibroblasts maintained in suspension suggesting that loss of the FN adhesion system is not sufficient to induce anchorage-independent growth. In conclusion, caution must be taken in assessing an up-regulation or down-regulation of integrin receptors when using immunohistochemistry or when comparing different staining techniques or different cell types.

Several integrins are known to bind to the RGD sequence with varying affinity including the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrin receptors and numerous commercially available peptides can inhibit this adhesion with varying efficiency. In our system, the linear GRGSPK peptide was not efficient for the murine $\alpha_5\beta_1$ integrin, but did produce almost 100% inhibition of adhesion to VN which is mediated through the $\alpha_v\beta_3$ receptor (data not shown). This result excludes the role of the $\alpha_v\beta_3$ in adhesion of SP1 cells to FN. It is expected that a longer peptide would have been required for inhibition of $\alpha_5\beta_1$-FN interaction in our system because the conformation of the peptide as well as the flanking regions which contain the synergy region are required for adequate inhibition (Hocking et al., 1994). It is also possible that the $\alpha_3\beta_1$ is involved in adhesion to FN. We did not assess the role of the $\alpha_3\beta_1$ integrin in adhesion to FN because we do not have the required blocking antibody. However, our results demonstrating that the BMA5 almost completely inhibits adhesion to FN shows $\alpha_5\beta_1$ is the primary receptor for FN on SP1 cells.

We have shown that the $\alpha_2\beta_1$ integrin is upregulated under anchorage-independent

conditions. Integrin expression is regulated by a number of factors including ECM and growth factors. Expression of $\alpha_2\beta_1$ can also change when cells are maintained under anchorage-independent conditions as seen with A431 human epidermoid cells (Waleh *et al.*, 1994). Loss of adhesion or changes in cell shape may alter transcription of integrin genes directly or indirectly through up-regulation or down-regulation of growth factors. The role of the $\alpha_2\beta_1$ receptor is further explored in Chapter 5.

There is conflicting evidence on the role of FN in tumor cell growth. Some studies have shown that FN is reduced from the cell surface of transformed cells and that adhesion to FN is reduced on transformed fibroblasts (Dalton *et al.*, 1992). In contrast, Coucke *et al* (1992) have shown that pretreatment of melanoma cells with FN, increased their ability to colonize lung tissue in mice. These differences may be a result of differences between fibroblasts and epithelial cells, the number of receptors on the cell surface or the degree of malignancy of the cells. Our results demonstrating that partial removal of FN from serum reduces colony growth suggests that FN is a limiting factor in colony growth. These results suggest that FN adhesion (but not spreading) may be required for growth of SP1 colonies. Thus SP1 cells may represent an intermediate stage in anchorage-independent growth.

Some colonies are able to grow in reduced serum conditions. SP1 under anchorage-independent conditions can produce their own FN. We have previously shown that adherent SP1 cells produce FN, but synthesis and secretion are considerably downregulated when the cells are cultured under anchorage-independent conditions (Appendix I). Fibronectin may not have been completely removed from the serum, and cells which require low amounts of FN may be able to form colonies. Cells under

anchorage-independent conditions do not proliferate as rapidly as adherent cells. Thus although SP1 cells downregulate FN synthesis and secretion, the remaining production may be sufficient to maintain colony growth. Addition of purified bovine plasma FN added to FN partially depleted serum only partially reconstitutes colony growth, suggesting that other components required for colony growth may be attached to the FN and co-precipitated from the serum. Growth factors such as TGF-β and HGF have been shown to bind ECM (Lamszus *et al.*, 1996) and may be incorporated onto the FN. TGF-β, a likely candidate, is effective at extremely low concentrations (0.1 ng/ml) and may be difficult to detect by conventional methods. Further investigation is required to determine whether any growth factors are present on exogenous or endogenous ECM proteins.

The inhibitory effect of collagen on SP1 colony growth is likely the result of two possible mechanisms: The action may be indirect, by interfering with binding of FN to integrins on the cell surface. SP1 cells have few collagen receptors therefore no signals are generated by having collagen at the cell surface, where it may interfere with positive signal generated from FN. Alternatively, the action could be direct, as a result of collagen binding to specific integrin receptors which generate a negative growth signal such as that during cell differentiation. Further investigation is required to determine the mechanisms by which collagen inhibits SP1 colony growth. However results obtained in chapter 5 support the former possibility. We have noted some variation in the ability of collagen to inhibit SP1 colony growth. SP1 cells are a heterogenous population of cells and show heterogeneity in their adhesiveness to Coll I. It is possible that some cells respond positively to collagen while others are unaffected or negatively affected. The phenotype of SP1 cells may change slightly after being in culture for several weeks, hence a freshly

thawed stalk may respond differently to Coll I than an older culture of cells.

We have also noted considerable variation in the total number of colonies obtained between colony assays. Possible sources of variation are errors in pipetting a small number of cells (2000/plate). Colony growth is sensitive to agar concentrations. Concentrations above 0.36% are inhibitory to colony growth, especially at early stages. Evaporation of water from the agar during the 10-14 day incubation results in a concentration of the agar and salts in the medium and reduces colony growth. Addition of medium to the top of the agar frequently inhibits the top layer from sticking to the bottom layer and to the plate and there is risk of the top layer being lost or damaged during fixation and staining of the colonies. To obtain more consistent results between experiments, these technical difficulties must be addressed.

We demonstrated that SP1 cells adhere poorly to LM but we also show that LM marginally increases colony growth of SP1 cells. There are at least two possibilities for this observation. First, we have shown that cells under anchorage-independent conditions increase surface expression of the $\alpha_2\beta_1$ receptor which is a laminin and collagen receptor. It is possible that this increase is sufficient for anchorage-independent cells in agar to respond to LM and promote colony growth. In support of this observation, Lin and Bertics (1995) demonstrated that clones of mouse B83L fibroblasts which adhered to laminin showed increased colony forming ability when compared to clones which did not bind to laminin. In addition, laminin and some of its peptides have been associated with increased tumor cell growth and to increase migration and invasion of cells (reviewed in Fields, 1993). Second, it is possible that SP1 cells secrete proteases which cleave laminin and generate peptides which promote growth more efficiently than the native protein.

We have shown that both TGF-β and HGF could enhance colony formation of SP1 cells. TGF-β was shown to be more efficient than HGF. Interestingly, many of the studies performed with growth factors which increase anchorage-independent growth are performed in the presence of 10% FBS. We have seen this effect in low serum conditions (1% FBS). In support of our results, two reports have shown that HGF can increase colony growth of MDCK cells and small cell lung carcinoma (Uehara and Kitamura, 1992; Seckl et al., 1994). Both of these reports have used 10% FBS in the assay.

In conclusion, the results in this chapter suggest a positive regulatory role for FN, $\alpha_5\beta_1$, $\alpha_2\beta_1$ and TGF-β in SP1 colony growth. These data further suggest that changes in the transformed phenotype mediated by HGF are likely to be in the proliferation or mitogenic pathway, while those mediated by TGF-β may be through proliferation or modulation of ECM-integrin interactions. In addition, our results have shown that a number of extracellular factors including ECM proteins and growth factors can affect anchorage-independent growth in both a positive and negative manner.

CHAPTER 5

ADHESIVE AND GROWTH PROPERTIES OF SP1

CELL CLONES CL-12-H AND CL-24-L

5.1 Rationale and Objectives

We have provided evidence in chapter 4 that integrin-ECM interactions play a role in anchorage-independent growth of SP1 cells. However, SP1 cells are a heterogenous population of tumor cells, therefore individual clones may have different adhesive and growth properties. The unstable genotype of tumor cells may cause some drift in the phenotypes in a rapidly growing population of cells. It is difficult to properly assess the adhesive and integrin properties in a heterogenous population of cells.

Cells able to grow in agar show a correlation with tumorigenicity in mice (Freedman and Shin, 1975). Several of the mechanisms which allow proliferation under anchorage-independent conditions are discussed in this thesis but there still remain many questions about ECM-integin interactions under anchorage-independent conditions. Transformed cells become less adhesive and less dependent on exogenous factors for growth and often show an autocrine stimulation. However growth factor autonomy alone is not sufficient for anchorage-independent growth (Stackpole *et al.*, 1995). The matrix and adhesive requirements are also important but not well understood in transformed cells. For example, the collagen receptor, $\alpha_2\beta_1$, is expressed on both normal and transformed mammary cells. Expression of $\alpha_2\beta_1$ is generally reduced on transformed mammary cells but low levels remain on the cell surface. Over-expression of $\alpha_2\beta_1$ in transformed mammary cells is associated with a reversion of the malignant phenotype (Zutter *et al.*, 1995). These results suggests that the presence of collagen receptors on the cell surface may play a physiological role in

regulating metastasizing cells. For example, *in vivo* tumors often degrade their basement membrane and invade into the local stroma which is rich in ECM proteins such as Coll I. In addition, Howlett *et al.* (1995) have shown that $\alpha_2\beta_1$ is required for morphogenesis of normal mammary cells in collagen gels.

In the previous chapter, we have shown that SP1 cells maintained under anchorage-independent conditions express higher levels of the $\alpha_2\beta_1$ than those maintained on plastic. We have also shown that SP1 cells show heterogeneity in their adhesive potential to collagen type I. These findings raise the possibility that collagen binding capacity and $\alpha_2\beta_1$ integrin expression may be differentially expressed in SP1 subpopulations.

To further investigate the role of ECM proteins in anchorage-independent growth, we must first obtain a population of cells with uniform adhesive properties and integrin profile. As seen in chapter 4, ECM proteins have both positive and negative influences on colony growth of SP1 cells. It is possible that some of the cells within the heterogenous population of SP1 cells respond positively to ECM proteins while others respond negatively. Such a situation may result in a null effect of the ECM or inconsistant results between experiments. It should also be possible to isolate a population of SP1 cells which grow efficiently in agar and another population which does not grow in agar.

In this chapter our objectives are:

1. To establish SP1 clones with high and low colony forming ability in agar.

2. To investigate the integrin expression, adhesive and growth properties of the clones.

3. To determine whether any differences in ECM or integrins profiles are associated with the ability to form colonies in agar.

5.2 Selection of SP1 clones with variable adhesive and anchorage-independent growth properties.

SP1 cells are a heterogenous population of cells with differences in adhesive and anchorage-independent growth properties. There is variation in their morphological appearance in culture and when plated in agar, only a small percentage of SP1 cells form colonies in agar. There is also heterogeneity in the size of the colonies. We diluted SP1 cells at low concentrations and allowed single cells to grow in small islands on plastics. These islands were isolated and cultured for several passages before being assayed. Approximately 35 clones were tested for their adhesiveness to various ECM proteins and for colony growth in soft agar. Some of the results of the adhesive profile of clones are in Appendix II. The results showed little variability in adhesion to FN or VN between different clones but a high degree of variability in adhesion to LM, Coll I, and Coll IV. The SP1 clones also demonstrated a wide spectrum in their ability to grow in agar supplemented with 7% FBS. We selected two clones at opposing ends of the spectrum; Cl-12-H which grows very well in agar (approximately 40%) and forms large colonies with less variability in size and Cl-24-L which does not grow well in agar (approximately 2%) as compared to the parent cell line, SP1 (approximately 10%) (Figure 14). There is considerable variation among colony experiments (discussed in Chapter 4). These two clones were sub-cultured 30 times and assayed again. The anchorage-independent phenotype was very stable as determined by periodic agar assays.

The results in this section show that there is clonal heterogeneity in the potential for SP1 cells to grow anchorage-independently and that this phenotype is stable.

Figure 14. <u>Growth of SP1, Cl-12-H and Cl-24-L cells in agar</u>

The colony assay was performed as described in the Material and Methods. SP1, Cl-12-H and Cl-24-L cells were harvested and assayed in agar supplemented with 7% FBS as previously described. All three cell lines were seeded at 2000 cells/plate. After 14 days, the plates were fixed as previously described and the colonies counted manually. The results below are expressed as the mean ± SD of quadruplicates. Figure 14 is a photograph of one of the plates.

A: SP1:   151 ± 15

B: Cl-24-L: 47 ± 8

C: Cl-12-H: 535 ± 31

A
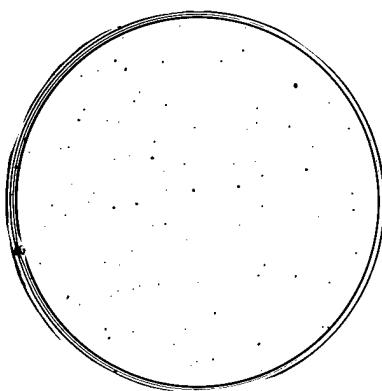
B
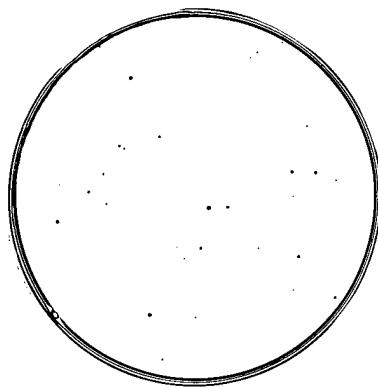
C
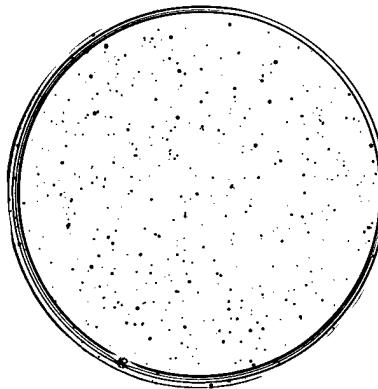
5.3 Cl-12-H cells grow efficiently in agar and in serum-free medium.

Our objective was to focus on differences in the adhesive properties of Cl-12-H and Cl-24-L and determine whether any observed differences correlated with their phenotypic differences in anchorage-independent growth. The morphology of Cl-12-H and Cl-24-L cells cultured on plastic in the presence of 7% FBS were very similar. The Cl-24-L cells show a more spread phenotype while Cl-12-H cells are more spindle shaped (Figure 15).

We compared the growth potential of the two clones with the parent population of SP1 cells. SP1 cells, Cl-12-H and Cl-24-L had similar growth rates when cultured in 7% FBS on tissue culture plastic as determined by ^{3}H-thymidine incorporation. However, in serum free conditions, the Cl-12-H clone incorporated significantly more ^{3}H-thymidine than either SP1 or the Cl-24-L cells (Figure 16).

Rahimi *et al.* (1996) have previously shown that HGF is produced by SP1 cells and that they express the c-Met receptor. To determine whether the increased growth of Cl-12-H was a result of increased HGF-c-Met interaction, we used suramin (a non-specific inhibitor of growth factor receptor-ligand interactions). The growth, as measured by ^{3}H-thymidine incorporation, of all three cell lines was inhibited by suramin (500 µg/ml) in serum-free medium (data not shown) (discussed further in discussion). We examined the HGF receptor status on SP1 cells and the two clones by immunoprecipitation and western blot analysis. We did not observe any difference in HGF receptor expression and tyrosine phosphorylation on SP1, Cl-12-H and Cl-24-L cells (data not shown).

The results in this section show that Cl-12-H are morphologically different than Cl-24-L cells and grow better in serum free conditions when adherent.

Figure 15. <u>Morphology of Cl-12-H and Cl-24-L on plastic</u>

Cl-12-H and Cl-24-L cells were seeded in 100 x 15 mm tissue culture plates in the presence of 7% FBS. After 8 h, the cells were photographed using a Leica DM IL inverted microscope using Kodak technical pan film (ESTAR-AH base). Magnification: 130 X
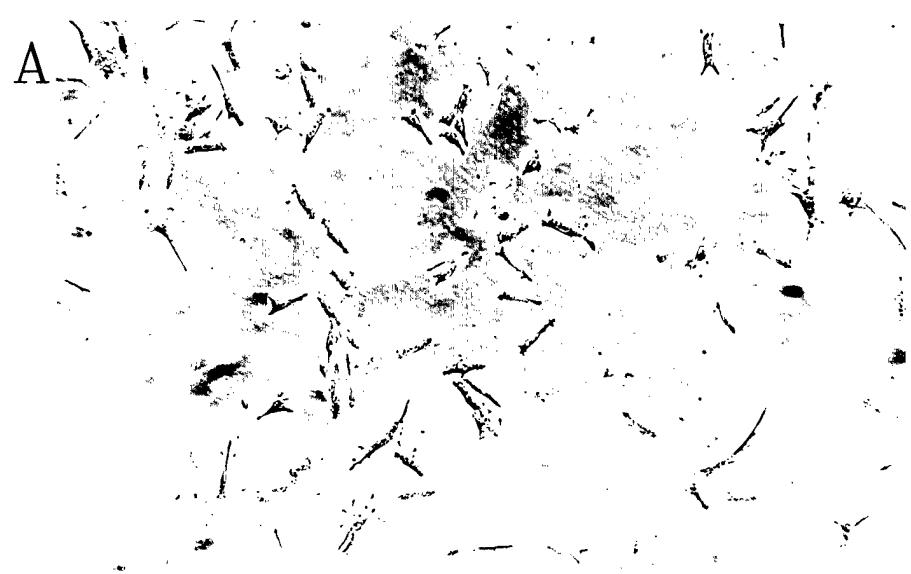
A: Cl-12-H

B: Cl-24-L

Figure 16. <u>Growth of SP1, Cl-12-H and Cl-24-L cells on plastic in serum-free conditions and</u> <u>7% FBS</u>

SP1 (empty bars), Cl-12-H (solid bars) and Cl-24-L cells (hached bars) were seeded (10,000/well) in 24 well tissue culture plates in the test medium (7% FBS or serum-free). After 24 h, 0.2 μCi ^{3}H-thymidine was added to each well and incubated for another 24 h at 37°C. The cells were harvested with trypsin-EDTA and placed in 96-well microtiter plates then transferred to filters using a cell harvester. ^{3}H-thymidine incorporation was measured (CPM/well) in a scintillation counter using Ecolume scintillation fluid. The results are expressed as the mean ± SD of four experiments; each experiment contained quadruplicate samples. ✱ indicates a significant increase in growth compared to SP1 and Cl-24-L cells grown under serum-free conditions ($p < 0.05$).

5.4 Cl-12-H cells adhere to collagen type I and express $\alpha_2\beta_1$

We investigated the adhesion profile of Cl-12-H and Cl-24-L cells. Cl-12-H and Cl-24-L cells adhere to a similar extent on FN and VN (data not shown). In contrast, Cl-12-H was able to adhere and spread on Coll I much more efficiently than Cl-24-L (Figure 17A and B). Cl-12-H cells were also able to adhere better to LM than SP1 or Cl-24-L cells (data not shown). Interestingly, both clones were able to adhere relatively well and to the same extent to Coll IV. As with SP1 cells, we tested the dependence of Cl-12-H and Cl-24-L adhesion on divalent cations. Both cell lines required either Ca^{2+}, Mg^{2+}, or Mn^{2+} to adhere to FN. Cl-12-H also required divalent cations to adhere to Coll (data not shown). None of the divalent cations could promote Cl-24-L cells to adhere to Coll I above baseline levels (data not shown).

We have shown that Cl-12-H cells adhere well to Coll I while Cl-24-L cells do not, therefore we investigated the expression of Coll receptors on the cell surface of the two clones. The primary integrin receptors for Coll are $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$. Immunoprecipitation of the integrin receptors for Coll using anti-α_2, α_3 and β_1 antibodies showed that Cl-12-H cells expressed higher levels of the $\alpha_2\beta_1$ integrin than Cl-24-L cells. Both cell lines expressed similar levels of the β_1 and the α_3 integrin (Figure 18A). In the α_2 immunoprecipitation we observed a band at approximately 110 kDa which co-migrates with the β_1 subunit but disappeared upon reduction of the samples (Figure 18B). We also observed a third band at approximately 100 kDa which is also present in the IgG control. This band was also lost after treatment with 2-mercaptoethanol. We do not have any antibodies which recognize the murine α_1 integrin subunit. However the α_1 subunit is 180 kDa in size compared to the other α subunits which are 120-130 kDa in size. Based on this

difference, the α_1 subunit can be distinguished from the other α subunits in the β_1 immunoprecipite. We did not observe any α_1 integrin subunit in the β_1 immunoprecipite. We have not determined whether $\alpha_2\beta_1$ is the primary receptor for collagen or what is the contribution of other CoII receptors such as $\alpha_3\beta_1$ and $\alpha_v\beta_3$ since we do not have any specific blocking antibodies or peptides to these mouse integrin receptors.

The results in this section show that Cl-12-H cells express higher levels of the collagen/laminin receptor $\alpha_2\beta_1$ and adhere well to collagen type I and laminin as compared to SP1 and Cl-24-L cells.

Figure 17. Adhesion of Cl-12-H and Cl-24-L cells to collagen

Panel A

The cell adhesion assay was performed as described in the Materials and Methods.

Linbro 96-well tissue culture plates were precoated with collagen types I and IV coated at

the concentrations indicated (obtained by two-fold serial dilution).   After 45 min at 37°C

the cells were fixed and stained as described in the Materials and Methods. The results are

expressed as the mean of duplicate samples. The experiment was repeated twice with similar

results.

Panel B and C. Photographs of Cl-12-H and Cl-24-L on collagen type I

SP1 cells were harvested and seeded in Linbro 96-well tissue culture plates (30,000

cells/well coated with collagen type I (10 µg/ml). The cells were allowed to adhere for 30

min then photographed on a Lieca DM IL inverted microscope using technical pan film.

Magnification: 120 X

B: Cl-12-H on collagen type I

C: Cl-24-L on collagen type I

A
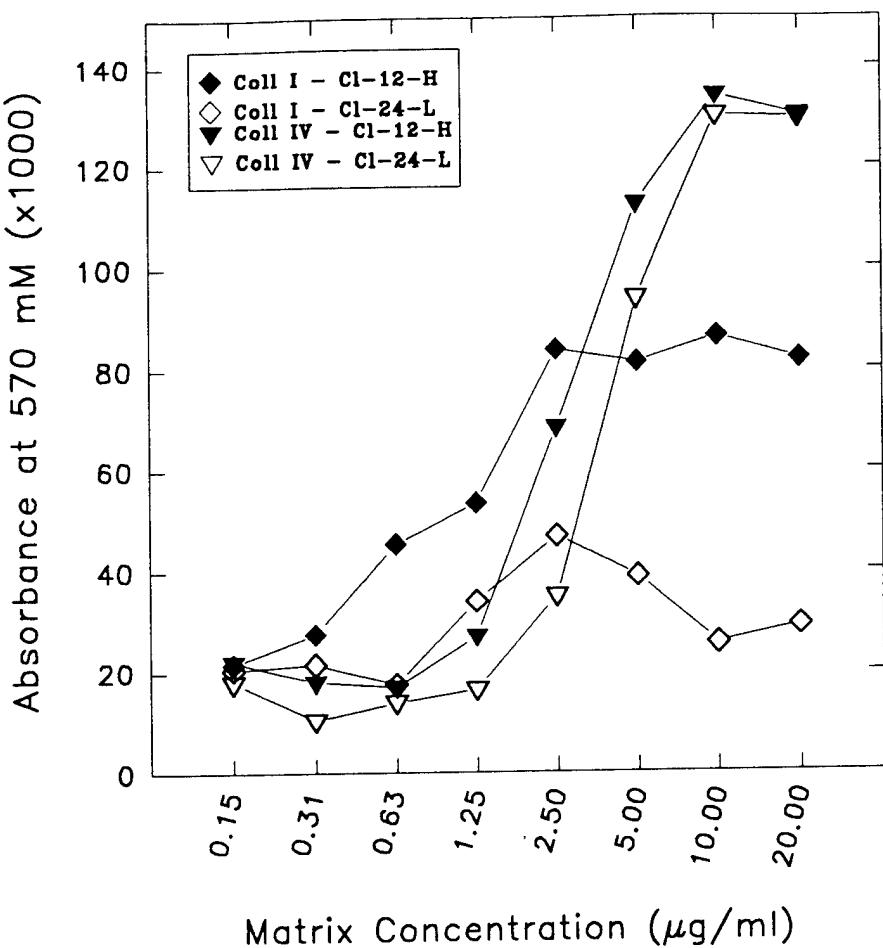
B

C
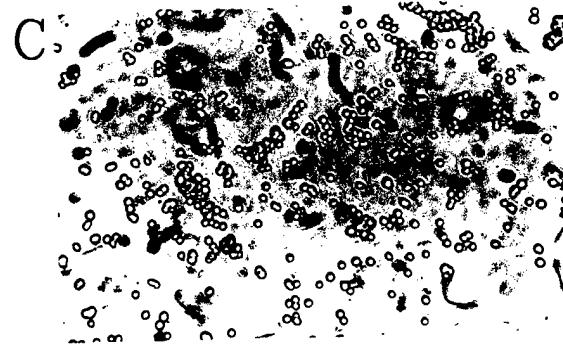
Figure 18. Collagen receptors on Cl-12-H and Cl-24-L cells

Panel A

Cl-12-H and Cl-24-L cells were harvested and labelled with a biotinylation reagent to biotinylate all cell surface proteins in PBS as described in the Materials and Methods. The cells were counted and the cell number normalized in each group then lysed in RIPA lysis buffer. The lysate were centrifuged and the protein concentration of the supernatant normalized and aliquoted into 4 groups. The integrins were immunoprecipitated with rabbit anti-mouse IgG (control), rabbit anti-β_1, anti-α_2 and anti- α_3 IgG and resolved by 7.5% SDS-PAGE under non-reducing conditions. The proteins were transferred to nitrocellulose and a western blot analysis performed using a streptavidin-horse radish peroxidase-conjugated antibody for detection. The proteins were visualized using ECL reagents. The vertical arrows ↓ show the position of the α_2 and α_3 subunits of Cl-12-H cells.

Panel B

An aliquot of the same immunoprecipitate as in Figure 18A was run under reduced conditions (5% 2-mercaptoethanol) and resolved by 7.0% SDS-PAGE. The proteins were transferred to nitrocellulose and a western blot analysis performed using a streptavidin-horse radish peroxidase-conjugated antibody for detection. The proteins were visualized using ECL reagents.

A

CI-12-H   CI-24-L

IgG β₁ α₂ α₃ IgG β₁ α₂ α₃ kDa

- 207

α →
β →

- 106

- 68

B

α₂   α₂   kDa

- 207

α₂→
β₁²→

- 106

- 68

CI-12-H CI-24-L

114

5.5 Fibronectin and collagen type I promote anchorage-independent growth of Cl-12-H cells

We have previously shown that FN can marginally increase colony growth of SP1 cells in soft agar in the presence of 1% FBS, while Coll I inhibits this growth. We tested the effect of FN, LM, VN and Coll I on colony growth in agar of Cl-12-H and Cl-24-L cells. Similar to the results observed with SP1 cells, FN and LM were found to promote colony growth of Cl-12-H cells. When added in combination, FN + LM or FN + Coll I showed an additive increase in colony growth (Figure 19A). Interestingly, collagen type I which inhibits colony growth of SP1 cells promotes that of Cl-12-H cells and could also increase colony growth in the presence of 7% FBS in a concentration dependent manner (Figure 19B). Neither FN or Coll I could affect growth of Cl-24-L cells in agar (Figure 19B).

In conclusion, we have isolated an SP1 clone which is very efficient at anchorage-independent growth, as well as growth in serum-free conditions. Cl-12-H cells also adhered to Coll I and expresses $\alpha_2\beta_1$ integrin. A summary of the properties of Cl-12-H, Cl-24-L and SP1 cells are presented in Table 3.

The results in this section show that both fibronectin and collagen type I increase colony growth of Cl-12-H cells.

Figure 19. <u>Colony growth of Cl-12-H cells in the presence of exogenous ECM proteins</u>

Panel A

The colony assay was performed as described in the Materials and Methods. The control group contains 1% FBS. The treatment groups also contain ECM proteins indicated on the X axis at a final concentration of 10 µg/ml before solidification. After 14 days, the plates were fixed and stained as previously described. The results are expressed as the mean ± SD of quadruplicates.

Panel B

The colony assay was performed as described in the Materials and Methods. SP1, Cl-12-H and Cl-24-L cells were seeded (2000/dish) in the presence of 7% FBS. Treatment groups also contained FN or collagen type I at 2.5, 5, or 10 µg/ml.

A

B

Cl-12-H   Cl-24-L

117

Table 3. <u>Growth and adhesive properties of SP1, Cl-12-H and Cl-24-L cells</u>

	SP1	Cl-12-H	Cl-24-L
Growth in 7% (plastic)	+++	+++	+++
Growth in serum-free medium	+	++	+
Adhesion to collagen type I	+	+++	-
Adhesion to fibronectin	+++	+++	+++
Adhesion to laminin	+	+++	+
Expression of $\alpha_2\beta_1$	+	++	-
Colony growth (7%)	++	+++	+

<u>Legend</u>:

Table 3 illustrates the properties of the parent SP1 cells and the SP1 cell clones, Cl-12-H and

Cl-24-L in growth, colony and adhesion assays.

-        not detectable

+   marginal growth or adhesion

++   moderate growth or adhesion

+++   Maximal growth or adhesion

118

5.6 <u>Discussion</u>

In this chapter we have shown that

1. SP1 cell clones with both high (Cl-12-H) and low (Cl-24-L) colony efficiency in agar can be isolated from the parent population, and agar colony forming ability is a stable phenotype in these cells.

2. The Cl-12-H clone adheres to collagen type I, expresses $\alpha_2\beta_1$ and grows better in serum free medium than SP1 cells or the Cl-24-L clone.

3. Fibronectin and collagen type I both increase colony growth of Cl-12-H cells.

We have demonstrated a correlation between the expression of $\alpha_2\beta_1$ integrin and SP1 colony growth. Our results, show that $\alpha_2\beta_1$ is increased in expression on the Cl-12-H clone. However, colony growth is associated with an increase in the transformed phenotype. Zutter *et al.* (1995) have shown that an increase in $\alpha_2\beta_1$ correlates with a decrease in the malignant phenotype. In untransformed cells $\alpha_2\beta_1$ is required for mammary gland morphogenesis and is involved in collagen gel contraction.

Cells receive many extracellular signals from the environment which affect their responses to signals from the $\alpha_2\beta_1$ integrin. It is possible that $\alpha_2\beta_1$-Coll interaction will cause growth inhibition and stimulate differentiation in one cell line but stimulate growth in another cell type. As previously mentioned with the subunit α_5, the level of expression, the degree of malignancy, and the tissue microenvironment are likely to contribute to the difference between our results and those obtained by others (discussed further in main discussion).

Cl-24-L clone does not increase colony growth in response to FN, suggesting that

ECM proteins may play a minor role in colony growth. We have shown that matrix assembly is also required for optimal colony formation, therefore it is possible that Cl-24-L cells lack non-integrin receptors involved in matrix organization, and that this property limits colony forming ability. It is also possible that CoII matrix assembly could promote anchorage-independent growth of Cl-24-L cells if they expressed the collagen receptor $\alpha_2\beta_1$ as does Cl-12-H cells. To test this hypothesis we are transfecting the Cl-24-L clone with the α_2 construct in collaboration with B. Chan. We will then test the ability of α_2 expressing cells to form colonies in soft agar in the presence of CoII. There is also the possibility that the α_2 and the α_5 integrin subunits may be acting together to mediate colony growth. Dzamba *et al* (1993) have shown that CoII I binding to FN promotes FN fibril assembly by an unknown mechanism. It is possible that FN is sufficient for matrix assembly or that collagen may increase matrix assembly in a cooperative way with FN.

There are other factors which we have not investigated that may participate in the anchorage-independent growth of Cl-12-H cells. It was shown that the proteoglycan syndecan I can regulate anchorage-independent growth. Mammary carcinoma cells transfected with syndecan I show a reduced ability to form spheroids in agar (Leppä *et al.*,1992; Numa *et al.*, 1995). Down-regulation of syndecan I by antisense resulted in an increased ability of NMuMg cells to form colonies in agar (Kato *et al.*, 1995). Experiments are underway to determine the level of syndecan I expression on the surface of SP1, Cl-12-H and Cl-24-L cells.

Our results demonstrated that Cl-12-H cells grows more efficiently in serum-free conditions. This finding suggests that Cl-12-H cells may secrete autocrine factors such as growth factors which may promote its growth under anchorage-independent conditions. We

used suramin which is thought to be a general inhibitor of growth factor-receptor interaction (Larsen, 1993; Coffey *et al.*, 1987). Suramin, which has been reported to inhibit growth of breast carcinoma cells (Vignon *et al.*, 1992; Foekens *et al.*, 1993), was able to inhibit the enhanced growth of Cl-12-H cells in serum-free conditions. However suramin is also an inhibitor of several enzymes such as DNA and RNA polymerases, protein kinase C, and DNA topoisomerase II (Bojanowski *et al.*, 1992; Ono *et al.*, 1988). It is not known whether suramin inhibits these enzymes by inhibiting receptor-ligand interactions or by other mechanisms, therefore caution is required in interpreting the results.

HGF is secreted by SP1 cells and is a likely candidate to be involved in Cl-12-H proliferation. We did not find any difference in HGF receptor expression or tyrosine-phosphorylation between the Cl-12-H and Cl-24-L cells. These results do not exclude the possibility that HGF and c-Met are involved in the increased growth of Cl-12-H in serum-free conditions. We have not yet investigated the amount of HGF secreted in conditioned medium due to the absence of a good antibody to murine HGF. We have also not investigated the rate of synthesis and endocytosis of c-Met or HGF in these cells nor the activation of several downstream signaling molecules. To determine whether c-Met is involved in the increased growth rate of Cl-12-H, we would use soluble Met-IgG fusion preotein which binds to soluble HGF making it inaccessible to the receptor on the cell surface. We are also investigating the possibility of an HGF intracrine loop. Further experiments are required to determine whether Cl-12-H cells secrete more HGF or whether activation of HGF receptor on the Cl-12-H and Cl-24-L is differentially affected under anchorage-independent conditions.

In conclusion, isolation of Cl-12-H and Cl-24-L clones enables us to study a number

of positive and negative regulators of anchorage-independent growth. We can study factors and conditions which inhibit anchorage-independent growth with the Cl-12-H clone and factors and conditions which promote anchorage-independent growth of the Cl-24-L clone. Our interests lie in the role of ECM-integrin interactions. These cells could also be used in other areas of study such as oncogene expression and cell cycle analysis. These clones may provide valuable information about the mechanisms which regulate anchorage-independent growth of transformed cells.

We have shown that ECM and growth factors affect anchorage-independent growth of SP1 cells but we do not know if these factors play a role in the initial cell survival required before colony growth. In the following chapter we will investigate the role of ECM protein and growth factors in SP1 cell survival.

CHAPTER 6

SURVIVAL OF SP1 CELLS UNDER ANCHORAGE-INDEPENDENT CONDITIONS. A ROLE FOR PI 3-KINASE

6.1 Rationale and Objectives

In order for tumor cells to metastasize they must detach from the primary tumor, enter the circulation where they are under anchorage-independent conditions for some time before they extravasate and grow at a distant site. One of the first obstacles the tumor cell encounters under anchorage-independent conditions is the need for survival. Most tumor cells injected in the blood stream of animals do not survive (Weiss *et al.*, 1982). It is thought that most die as a result of the loss in adhesiveness. The tumor cells must survive before they can grow, proliferate and form a metastatic lesion. Extracellular matrix proteins and growth factors are key regulators of cell survival and growth under adherent conditions. Cells deprived of serum or maintained under anchorage-independent conditions often undergo cell death via apoptosis (Frisch and Francis, 1994). Adhesion to ECM proteins via integrins has been shown to mediate survival of adherent cells (Ruoslahti and Reeg, 1994; Zhang *et al.*, 1995). Basement membrane proteins, provided by Matrigel, were required for suppression of apoptosis in mammary epithelial cells (Boudreau *et al.*, 1995, 1996). Adhesion to FN via $\alpha_5\beta_1$ was required for survival of CHO cells in serum free-conditions (Zhang *et al.*, 1995). Ligation of $\alpha_v\beta_3$ was required for survival of human melanoma cells in a three-dimensional collagen gel (Montgomery *et al.*, 1994). These results provide evidence that occupancy of integrin receptors provides a cell survival signal.

A number of growth factors, including EGF, PDGF, and NGF can also promote cell survival by a PI 3-kinase-dependent pathway (Yao and Cooper, 1995, 1996). In

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addition, we have previously shown that PI 3-kinase is required for the HGF-mediated mitogenic response on adherent SP1 cells (Rahimi *et al.*, 1996b).

Very little is known about the role of ECM proteins and growth factors in tumor cell survival under anchorage-independent conditions. It is known that soluble FN can bind to the surface of fibroblasts and hepatocytes in suspension (Akiyama and Yamada, 1985; Johannson, 1985). Soluble VN was not sufficient to inhibit apoptosis in normal endothelial cells (Re *et al.*, 1994). Although some tumor cells have bypassed the requirement for adhesion, it is not known whether ECM proteins still function in providing a growth or survival signal when under anchorage-independent conditions.

In order to determine some of the factors which promote survival of SP1 cells we developed a survival assay using SP1 cells maintained under anchorage-independent conditions. In the previous chapters we have shown that SP1 cells require both ECM proteins and growth factors for adequate colony formation. We now investigate whether ECM proteins and growth factors are required for survival of SP1 cells under anchorage-independent conditions.

In this chapter, our objectives are:

1. To determine whether FN can promote survival of SP1 cells via ligation of $\alpha_5\beta_1$ receptor under anchorage-independent conditions.

2. To determine whether growth factors such as HGF and TGF-β are able to promote survival of SP1 cells.

3. To establish whether PI 3-kinase is involved in ECM and HGF mediated survival.

4. To determine whether FN and HGF can mediate protein-tyrosine phosphorylation of PI 3-kinase and FAK under anchorage-independent conditions.

6.2 HGF, TGF-β, and FN promote survival of SP1 cells

We have shown in chapter 4 that FN, HGF, and TGF-β can increase colony formation of SP1 cells in agar. We now investigate whether FN, HGF and TGF-β can also promote survival of SP1 cells in suspension.

Preliminary experiments were performed to determine the optimal concentration of cells, volume of medium and serum concentations in the survival assay. We chose to perform the assay in small plates (35 x 10 mm) to reduce the volume and to save on reagents. We needed a sufficient number of cells to count, however if too many cells are added to the plate they condition the medium within the experimental time and cell survival is increased. In addition, if too many cells are used they tend to aggregate and cluster, which also increases cell survival. We needed to maintain the cells in single cell suspension as much as possible. We chose 1% FBS because it maintained a reasonable survival rate in the tested time period. Under serum free conditions, approximately 95% of the cells died after 24 h and we could only observe increases in cell survival. In the presence of 7% FBS, approximately 95% of the cells survived after 24 h and we could only observe decreases in cell survival. Using 1% FBS yielded a cell survival of 70-80% and permitted us to observe both increases in cell survival and death. We chose a 24 h time point to avoid effects resulting from the cells conditioning the medium.

We have shown that HGF at concentrations between 5-30 ng/ml can promote cell survival and that TGF-β at concentrations between 0.1-0.5 ng/ml is sufficient to promote survival of SP1 cells maintained in suspension. Concentrations of HGF higher than 30 ng/ml and TGF-β higher than 0.5 ng/ml resulted in a decrease in cell survival. Fibronectin

could promote survival at concentrations greater than 1.0 μg/ml. Concentrations of FN

greater than 10 μg/ml did not improve or reduce survival beyond the maximal effect of a

50% increase in survival (Figure 20A). In contrast to FN, Coll I and LM did not promote

survival of SP1 cells under anchorage-independent conditions (Figure 20B). The 70 kDa

fragment of FN which inhibits fibril formation but does not interfere with cell adhesion,

could not promote survival and had no effect on survival mediated by FN (data not shown).

This result suggests that fibril formation is not required for survival under anchorage-

independent conditions.

The results in this section show that fibronectin, HGF and TGF-β can promote

survival of SP1 cells under anchorage-independent conditions in a concentration-dependent

manner.

Figure 20. <u>Effect of FN, HGF and TGF-β on SP1 cell survival</u>

Panel A

The survival assay was performed as described in the Materials and Methods. SP1 cells were seeded (20,000/plate) in 1.5 ml RPMI 1640 supplemented with 1% FBS in agar coated plates (35 x 10 mm) for 24 h at 37°C. The control group contains 1% FBS only. The treatment groups contain FN, HGF or TGF-β at the indicated concentrations in addition to 1% FBS. The reagents were added at the start of the experiment. The cells were then harvested in eppendoff tubes, spun down at 8000 rpm for 2 min and resuspended in 50 μl RPMI containing 4 μg/ml acridine orange and 4 μg/ml ethidium bromide. The cells were counted (at least 100/group) under a Leitz fluorescence microscope using an I filter. The experiment was repeated twice with similar results.

Panel B

The survival assay was performed as described in the Materials and Methods. The control group (solid bar) contains 1% FBS. The treatment groups contain 1% FBS as well as FN, Coll I or LM at a concentration of 10 μg/ml. The results are expressed as the mean ± SD of 3 experiments. ✱ indicates a significant reduction in the percent survival compared to the control (p < 0.05).

A

B

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6.3 Ligation of $\alpha_5\beta_1$ promotes survival of SP1 cells

To determine whether the effect of FN on SP1 survival was mediated through the $\alpha_5\beta_1$ receptor, we tested the effect of BMA5 (anti-α_5 integrin blocking antibody) in our survival assay. The BMA5 antibody blocks adhesion by interfering with the $\alpha_5\beta_1$ integrin-ligand binding. However, a number of antibodies which bind to integrin subunits can cause conformational changes in the integrins which cause the generation of intracellular signals (activating antibodies), BMA5 is a newly developed antibody and inhibits $\alpha_5\beta_1$ mediated adhesion to FN (Fehlner-Grdiner *et al.*, 1996), but no data is yet available on whether it possess activating properties. The BMA5 antibody (hybridoma conditioned medium) could significantly promote survival of SP1 cells in a 24 h assay as compared to the 1% FBS control. The increase in survival observed with BMA5 was similar to that observed with FN. Addition of the affinity purified BMA5 antibody at a concentration of 10 μg/ml could also significantly promote survival of SP1 cells under anchorage-independent conditions, but was less efficient than BMA5 added as hybridoma conditioned medium (Figure 21). In contrast, a rabbit anti mouse IgG control or a non-blocking anti-α_5 antibody (5H10) which recognizes the extracellular domain of the α_5 integrin subunit but does not interfere with ligand binding did not affect survival (Figure 21). Interestingly, addition of a secondary rabbit anti-mouse IgG which promote capping and internalization of the primary BMA5 antibody, resulted in an increase in cell death.

The results in this section show that fibronectin mediated survival of SP1 cells under anchorage-independent conditions, occurs via the $\alpha_5\beta_1$ integrin.

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6.4 PI 3-kinase is required for FN + HGF-dependent cell survival

PI 3-kinase activity was shown to be required in HGF-mediated proliferation of adherent SP1 cells (Rahimi *et al.*, 1996) and is involved in nerve growth factor (NGF) mediated survival of adherent cells (Yao and Cooper, 1995 and 1996). We now investigated whether PI 3-kinase is involved in HGF and FN-mediated cell survival.

Individually, HGF and FN could promote cell survival by approximately 50% of the control (1% FBS), however when added in combination, HGF + FN resulted in an additive effect which was comparable to the survival observed with 7% FBS and significantly greater than FN or HGF alone (Figure 22). We used two approaches to determine whether PI 3-kinase is involved in survival of SP1 cells under anchorage-independent conditions.

First, wortmannin, an inhibitor of PI 3-kinase, increased cell death in all groups including 7% FBS. However, the additive effect of both FN + HGF on cell survival was strongly inhibited by wortmannin, resulting in a survival similar to HGF or FN alone, which were only slightly reduced as compared to untreated controls (Figure 22). TGF-β could also reduce cell death by approximately 50% and acted in an additive fashion with FN (data not shown). Secondly, we used Cl-12-H cells transfected with a dominant negative mutant of the p85 subunit of PI 3-kinase, designated Δp85 (obtained from M. Kasuga). The Δp85 protein lacks the binding site for the p110 catalytic subunit of PI 3-kinase. A plasmid containing the Δp85 cDNA was transfected into Cl-12-H using lipofectamine by Eric Tremblay and the transfected cells were characterized by Nader Rahimi. One of the clones isolated, designated C23, expresses high levels of Δp85, and shows reduced PI 3-kinase activity (Rahimi *et al.*, 1996). C23 cells showed no additive

effect on survival when HGF and FN were added together. The increased cell survival in response to HGF or FN alone was only slightly affected. In contrast, another transfected clone, designated C22, which does not express high levels of Δp85 and maintains PI3-kinase activity, retained an additive effect of HGF + FN on SP1 cell survival (Figure 23).

We tested the ability of the Δp85 mutants to form colonies in soft agar. Colony formation correlated with the level of expression of the mutant p85 subunit. The clone C23, expressing high levels of Δp85 was less efficient than the control Cl-12-H cells and the control transfectant C22 cells in forming colonies (Figure 24). Further experiments are required to ensure that the results observed with colony growth are not a result of clonal selection.

The results in this section suggest that PI 3-kinase is required for the additive effect of FN + HGF on SP1 cell survival and growth under anchorage-independent conditions.

Figure 21. <u>SP1 cell survival in the presence of BMA5 antibody</u>

The survival assay was performed as described in the Materials and Methods. The control group contained 1% FBS. The treatment groups contain 1% FBS in addition to the indicated antibodies. BMA5 (hybridoma conditioned medium) was added at 30% v/v (sup). Affinity purified BMA5 (conc) was added at 10 μg/ml. Rabbit anti-mouse IgG (Rb αM IgG) and a non-blocking rat anti-α_5 mAb, 5H10 (anti-α5), were added at 30 μg/ml at the start of the assay. The cells were stained as previously described. The results are expressed as the mean $\pm$ SD of 3 experiments. ✱ and ✱✱ indicates a significant reduction in the percent cell death as compared to the control (p < 0.05).

Figure 22. <u>Effect of wortmannin on FN and HGF-mediated cell survival of SP1 cells</u>

SP1 cells were seeded (20,000/dish) in 1.5 ml RPMI 1640 with 1% FBS on agar coated plates (35 x 10 mm) for 24 h at 37°C. The medium was supplemented with FN (10 μg/ml) or HGF (10 ng/ml) as indicated. Wortmannin (Wort) was added to some groups at a concentration of 100 nM at the start of the experiment. After 24 h the cells were then harvested and stained with 4 μg/ml acridine orange and 4 μg/ml ethidium bromide. The cells (at least 100/group) were counted under a fluorescence microscope using an I filter. The results are expressed as the mean $\pm$ SD of four experiments. * indicates a significant reduction in the percent cell death as compared to the 1% FBS control ($p < 0.05$). ** indicates a significant reduction in the percent cell death as compared to the FN or HGF-treated groups ($p < 0.05$).

Figure 23. <u>Effect of FN and HGF on cell survival on Δp85 transfectants</u>

Two SP1 cell clones transfected with Δp85, designated, C22 and C23, were seeded (20,000/dish) in 1.5 ml RPMI 1640 with 1% FBS on agar coated plates (35 x 10 mm) for 24 h at 37°C. Treatment groups contained FN (10 μg/ml) or HGF (10 ng/ml) or FN + HGF together. The cells were then harvested, stained and washed as described in the Materials and Methods. The results are expressed as the mean $\pm$ SD of four experiments. ✱ indicates a significant reduction in the percent cell death as compared to the 1% FBS control ($p < 0.05$). ✱✱ indicates a significant reduction in the percent cell death as compared to the FN or HGF-treated groups ($p < 0.05$).

Figure 24. <u>Colony efficiency of Δp85 transfectants</u>

The agar assay was performed as described in the Materials and Methods. The Cl-12-H, C22 and C22 Δp85 mutants were all seeded at 2000 cells/dish in 7% FBS. After 14 days, the plates were fixed and stained as described in the Materials and Methods. The results are expressed as the mean ± SD of quadruplicates. The experiment was repeated 3 time with similar results.
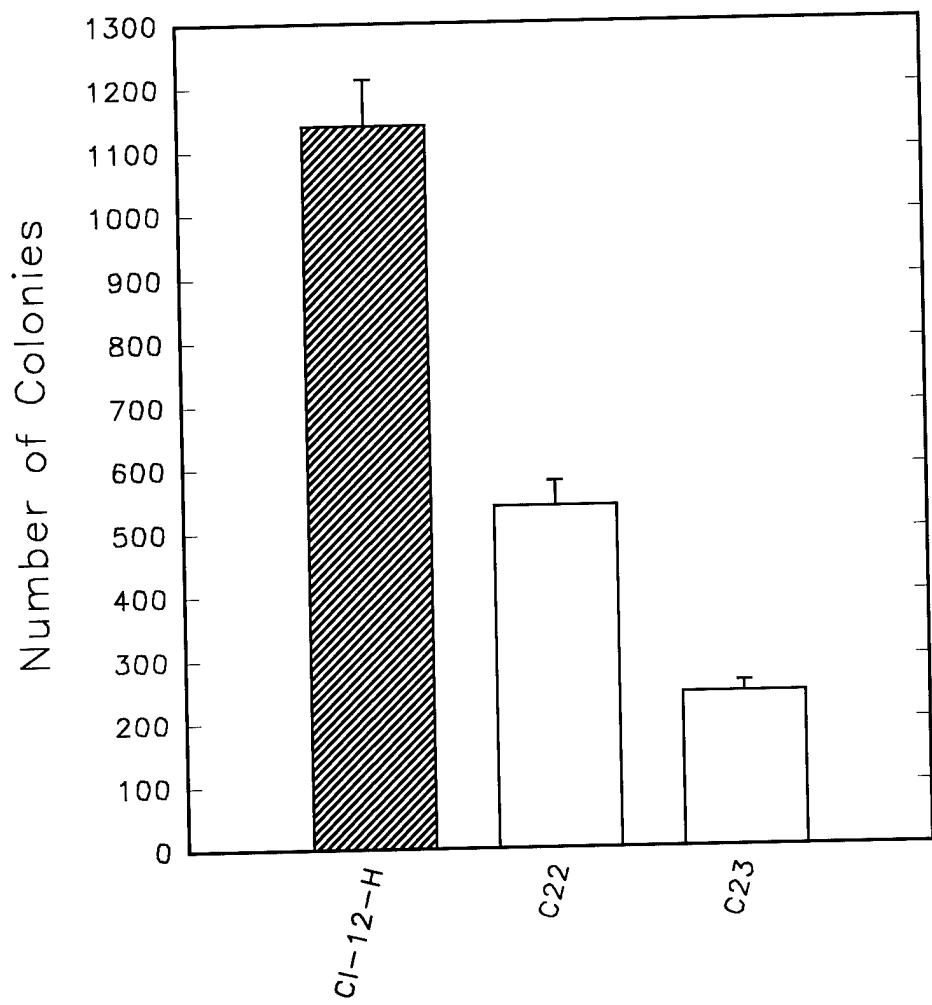
6.5   Tyrosine phosphorylation of the HGF receptor is reduced under anchorage-independent conditions

We have previously demonstrated that HGF is a strong mitogen for SP1 cells under adherent conditions and that HGF and HGF receptor are co-expressed on SP1 cells (Rahimi *et al.*, 1996). We have also shown that HGF can increases colony growth of SP1 cells in agar (Chapter 4). SP1 cells show continuous growth in serum-free medium consistent with an autocrine loop. We investigated the tyrosine phosphorylation of the HGF receptor under both adherent and anchorage-independent conditions. HGF receptor was consititutively phosphorylated on tyrosine residues in adherent SP1 cells. We observed only a slight increase in tyrosine phosphorylation upon addition of exogenous HGF at a concentration of 20 ng/ml. Tyrosine phosphorylation of HGF receptor was reduced under anchorage-independent conditions, but was restored in the presence of 20 ng/ml exogenous recombinant HGF (Figure 25A). The decrease in tyrosine phosphorylation was not a result of protein loading since both lanes contained equal amounts of HGF receptor (Figure 25B) When the blot was probed with anti-c-Met both the p145 c-Met protein and the p190 precursor were detected (Figure 25B), however only the p145 protein was tyrosine phosphorylated (Figure 25A).

6.6   FAK is poorly tyrosine phosphorylated under anchorage-independent conditions

Adhesion of many cell lines to ECM proteins results in the tyrosine phosphorylation of FAK, which is an important component in focal adhesion assembly and signal transduction pathways (Plopper *at al.*, 1995). We also observed that adhesion of SP1 cells to FN caused tyrosine phosphorylation of FAK which is increased in the presence of HGF,

as reported by others (Matsumoto *et al.*, 1994). In contrast, soluble FN, HGF or FN + HGF together were unable to induce FAK phosphorylation above that observed in untreated SP1 cells maintained in suspension (Figure 26). We attempted to reprobe the blot with anti-FAK to check the FAK content in each lane, however our anti-FAK antibody was unable to detect the protein by western blotting analysis. However, we did normalize the number of cells and the protein concentration in the lysate before immunoprecipitation.

The results in these two sections show that tyrosine phosphorylation of the HGF receptor and FAK are reduced under anchorage-independent conditions.

Figure 25. HGF receptor tyrosine phosphorylation under adherent and anchorage-independent conditions

Panel A

SP1 cells were prestarved overnight in RPMI 1640 medium. Six h prior to harvesting some SP1 cells were harvested and plated on non-tissue culture plastic coated with BSA. Prior to harvest (30 min) some groups were treated with 20 ng/ml recombinant HGF. Cells were harvested using 5 mM EDTA and lysed in 1% NP40 lysis buffer, immunoprecipitated with anti-c-Met, analyzed by 6.0% SDS-PAGE and transferred to nitrocellulose followed by western blotting. The blot was immunoblotted with anti-phosphotyrosine antibody and the bands visualized using ECL reagents. The experiment was repeated twice with similar results.

Panel B. The same blot as in panel A was stripped (100 mM TRIS, 4% SDS, at 50°C for 30 min) and reprobed with anti-c-Met. The proteins were visualized using ECL reagents.

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A

Adherent | Non - Adherent

Control | HGF | Control | HGF

KDa
- 214

p145^met →

- 111

- 74

IP: α-met
Blot: α-PY

B

Adherent | Non - Adherent

Control | HGF | Control | HGF

KDa
- 214

p145^met →

- 111

- 74

IP: α-met
Blot: α-met

Figure 26. Western blot analysis of FAK phosphorylation under adherent and anchorage-independent conditions

SP1 cells were prestarved in RPMI 1640 for 24 h, harvested and replated on Poly-L-lysine (PLL) (1:10 dilution) or FN-coated plates (10 μg/ml) for adherent groups (FN). One adherent group was also treated with HGF (20 ng/ml) (FN + HGF). The anchorage-independent (AI) groups were incubated in 10 ml tubes with FN (40 μg/ml) or HGF (20 ng/ml) or a combination of both for 30 min at 37°C. The cells were lysed in 1% NP-40 lysis buffer and the total protein concentration standardized in each group. FAK was immunoprecipitated with anti-FAK mAb. The immunoprecipitates were washed in lysis buffer and resolved by 8.0% SDS-PAGE. The proteins were transferred to nitrocellulose and blotted with anti-phosphotyrosine antibody then, visualized by ECL.

Adherent | Non-Adherent

PLL | FN | FN+HGF | AI | +FN | +HGF | +FH+HGF

p125 →

IP: anti-FAK
Blot: anti-PY

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6.7 Tyrosine phosphorylation and activity of PI 3-kinase

The biological effect (colony growth and survival) of FN and HGF on SP1 cells under anchorage-independent conditions implies that intracellular signals are transduced from these interactions. We investigated the tyrosine phosphorylation states of several candidate signaling molecules. PI 3-kinase is constitutively phosphorylated in SP1 cells adherent to poly-L-lysine and on FN, and showed little change in the level of tyrosine phosphorylation in response to HGF (Figure 27). Under anchorage-independent conditions PI 3-kinase showed a low level of tyrosine phosphorylation. We did not observe any consistent increase in PI 3-kinase phosphorylation in response to FN, HGF, or FN + HGF under anchorage-independent conditions. Preliminary results of SP1 cells grown under anchorage-independent conditions demonstrated that PI 3-kinase activity was increased in a stepwise manner in cell lysates of cells treated with FN, HGF, and FN + HGF compared to untreated cells (Figure 28).

In conclusion, the results in this chapter demonstrate a role for PI 3-kinase in the additive effect of FN and HGF under anchorage-independent conditions.

Figure 27. <u>Western blot analysis of PI 3-kinase phosphorylation under adherent and anchorage-independent conditions</u>

SP1 cells were prestarved for 24 h, harvested and replated on Poly-L-lysine(PLL) or FN-coated plates (FN). One adherent group on FN was treated with HGF at 20 ng/ml (FN + HGF) The anchorage-independent (AI) groups were incubated in 10 ml tubes in the presence of FN (40 μg/ml) or HGF (20 ng/ml) for 60 min at 37°C. The cells were lysed in 1% NP-40 lysis buffer and PI 3-kinase was immunoprecipitated with anti-PI 3-kinase rabbit IgG. The immunoprecipitates were washed in lysis buffer and resolved by 8.0% SDS-PAGE under reducing conditions. The proteins were transferred to nitrocellulose and blotted with anti-phosphotyrosine antibody and visualized by ECL. The experiment was performed twice with similar results.

Panel A: blotted with anti-phosphotyrosine antibody

Panel B: blotted with anti PI 3-kinase

A

Adherent   Anchorage-independent

PLL   FN   FN +HGF   AI   AI+FN   AI+HGF   AI+FN+HGF

p85 →

IP: Anti-PI 3-kinase

Blot: Anti-PY

B

p85 →

IP: Anti PI 3-kinase

Blot: Anti PI 3-kinase

Figure 28. PI 3-kinase activity in response to HGF and FN under adherent and anchorage-independent conditions

SP1 cells were maintained anchorage-independently for 1 h at 37°C in the absence or presence of HGF (20 ng/ml) or FN (20 μg/ml) or both (FN+HGF) or 7% FBS. The control group is a sham group, which contains no immunoprecipitated proteins The cells were lysed in 1% NP40 lysis buffer and PI 3-kinase immunoprecipitated with anti-phosphotyrosine antibody. The immunoprecipitates were washed and incubated with the substrate phosphatidylinositol and 15 μCi gamma ^{32}P-ATP. After 20 min, the reaction was stopped with 6 M HCl and the lipids were extracted with chloroform/methanol and resolved by TLC. The plate was autoradiographed and the radioactive area corresponding to the radiolabelled phosphoinositol 3-phosphate (PI 3-P) was removed and measured in a scintillation counter.

6.8 <u>DISCUSSION</u>

In this chapter we have shown that:

1. Fibronectin, HGF and TGF-β can promote survival of SP1 cells under anchorage-independent conditions.

2. Fibronectin and HGF act cooperatively to promote cell survival.

3. PI 3-kinase is a key component in the fibronectin- and HGF-mediated additive effect on survival.

In this chapter, we have shown that both FN, and HGF can enhance survival of SP1 cells under anchorage-independent conditions. The BMA5 antibody could also promote cell survival suggesting that FN-dependent survival is mediated through the $\alpha_5\beta_1$ integrin. In support of our results, adhesion to FN via $\alpha_5\beta_1$ was required for survival of CHO cells (Zhang *et al.*, 1995). In contrast, FN or Coll could not promote survival of CID-9 mammary epithelial cells in serum free conditions. However a basement membrane matrix was able to suppress apoptosis, provided that a three-dimensional alveolar structure was present (Boudreau *et al.*, 1996). In addition, Montgomery *et al.* (1994) demonstrated that the $\alpha_v\beta_3$ integrin could promote survival of melanoma cells in a three-dimensional collagen matrix. They showed that the $\alpha_2\beta_1$ integrin mediated initial cell adhesion in the collagen gel but did not promote survival. The melanoma cells secreted proteases which digested the collagen, revealing a cryptic RGD site which can bind to the $\alpha_v\beta_3$ receptor. Further experiments are required to determine the role of the $\alpha_2\beta_1$ and $\alpha_v\beta_3$ in our system.

Addition of the secondary antibody rabbit anti-rat IgG should cause capping and endocytosis of the receptor and generate a signal through the $\alpha_5\beta_1$ integrin. Our results show that addition of the rabbit anti-rat IgG with BMA5 causes an increase in cell death

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as opposed to the expected increase in survival which is seen with BMA5 alone. There are at least 2 possible explanations for this result. (1) The addition of the primary and secondary antibody may cause rapid capping and endocytosis of the receptor resulting in continuous depletion of the receptor from the cell surface; thus no signal would occur. Varner *et al.* (1995) have shown that unligated $\alpha_5\beta_1$ generates a negative signal while ligated receptor generates a positive signal. Thus, in our experiment, continuous depletion of the receptor from the cell surface may mimic an unligated receptor. (2) Addition of BMA5 alone may cause a slow process of capping and endocytosis and thus a moderate signal is generated resulting in increased survival but the addition of the secondary antibody causes a greatly enhanced capping and endocytosis which results in a proliferation signal which the cell can not properly interpret in a spherical morphology and therefore may triggers apoptosis.

We have shown that the concentration of HGF required for survival is crucial. Concentrations of HGF which are mitogenic for SP1 cells under adherent conditions (30-60 ng/ml) cause rapid cell death under anchorage-independent conditions. We are currently determining the mechanism of cell death of SP1 cells treated with high concentrations of HGF under anchorage-independent conditions. These results suggest that adhesive interactions are required for proper transmission of the HGF signal. Higher concentrations of TGF-β also have a negative effect on cell survival.

PI 3-kinase is required for growth factor-mediated inhibition of apoptosis in adherent cells. In this chapter, we have shown that it is also required for survival in the presence of both FN + HGF. Most previous studies performed on growth factor mediated survival are on adherent cells, therefore both adhesion and growth factor signals are

present. In our system, we needed to add the adhesion signal in the form of soluble matrix, hence we see a phenomenon similar to adhesive conditions. In conclusion, for optimal HGF mediated survival, SP1 cells require the adhesion signal, as do adherent cells, and survival is dependent on PI 3-kinase. These results show that non-adherent SP1 cells can respond to environmental cues from soluble ECM and growth factors in a similar manner as adherent cells.

It has been shown that FAK is required for adhesion-dependent cell survival (Frisch *et al.*, 1996), and that PI 3-kinase is required for platelet-derived growth factor stimulated phosphorylation of FAK (Rankin *et al.*, 1996; Saito,Y. *et al.*, 1996). Hence FAK is involved in both integrin and growth factor mediated pathways. In our system, we did not observe an increase in FAK phosphorylation in response to FN or HGF under anchorage-independent conditions. These results suggest that survival is mediated by FAK-independent pathways or that FAK has a dual function in both cell spreading and signaling. It is also possible that signaling via FAK actually results as a consequence of cell spreading. Roskelley and Bissell (1995) demonstrated that FAK was phosphorylated in mammary cells in response to basement membrane proteins in a three-dimensional ECM overlay assay. It may be that Matrigel provides more structure to mammary cells than soluble FN and HGF, or that additional components are required for FAK phosphorylation. It is possible that a low level of activity is still present under anchorage-independent conditions, but is not apparent using western blotting techniques.

In conclusion, we have demonstrated using wortmannin and a dominant negative form of p85 subunit of PI 3-kinase, that PI 3-kinase is involved in FN + HGF mediated survival of SP1 cells under anchorage-independent conditions.

CHAPTER 7

GENERAL DISCUSSION

7.1 Impact on field

The results presented in this thesis have made 2 main novel contributions to the understanding of tumor cell growth under anchorage-independent conditions.

1. A number of reports have shown the presence of ECM proteins in tumor spheroids. However this is the first report where individual ECM proteins in spheroids were investigated directly. We show by partially removing FN from tissue culture medium with optimal growing conditions (7% FBS), and by adding FN to tissue culture medium with limiting growth conditions (1% FBS), that FN is required for anchorage-independent survival and growth of SP1 cells. In contrast, Coll I inhibited colony growth of SP1 cells which express very low levels of the Coll receptor $\alpha_2\beta_1$. The SP1 cell clone, Cl-12-H, which expresses higher levels of $\alpha_2\beta_1$ showed increased colony growth in response to Coll I. Thus, different ECM proteins are capable of promoting growth, providing the appropriate cell surface receptors are present and in sufficient numbers. Our results also show that ECM-integrin interactions alone can not induce anchorage-independent growth of SP1 cells. They can only modulate anchorage-independent growth in cells with the potential to grow under these conditions.

2. Previous work performed on the role of ECM proteins and growth factors in cell survival has been performed in adherent normal or transformed cells. This is the first report investigating the role of ECM protein and growth factors in tumor cells under anchorage-independent conditions. We have demonstrated a cooperative role for FN and HGF in cell survival under anchorage-independent conditions. In addition, we have shown

that PI 3-kinase is required in FN + HGF mediated survival under anchorage-independent conditions as demonstrated by using wortmannin, a PI 3-kinase inhibitor and cells transfected with a dominant negative p85 subunit of PI 3-kinase.

7.2 Perspective to discussion

In an attempt to investigate why some tumor cells do not require adhesion to the ECM to grow and proliferate, one quickly realizes that a large number of factors play a role. First, many cells do not survive when removed from their substratum and undergo apoptosis; therefore the issue of cell survival under anchorage-independent conditions arises. Secondly, the concept of proliferation brings in the question of regulation and progression through the cell cycle. Thirdly, one cannot investigate the role of ECM in anchorage-independent growth without an in depth understanding of integrins and their signaling mechanisms since they are the primary cell surface receptors for ECM proteins. Fourthly, cell adhesion to ECM proteins modulates the response of cells to growth factors and vice-versa. Finally, to add to the complexity, cell shape, degree of differentiation and the cell type are all determining factors in the ability of the cell to respond to ECM proteins and growth factors.

The following discussion will bring out some key concepts in the role of ECM and integrins in tumor cell adherent and anchorage-independent growth with emphasis on the mammary gland. I will also discuss some relevant work from others and bring the work presented in this thesis into context.

As previously discussed, tumor spheroids are an intermediate model between monolayer cultures and *in vivo* tumor nodules. In light of this model, Paulus *et al.* (1994)

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investigated glioblastomas in biopsy specimens, spheroids, and early monolayer cultures and observed changes in both integrin and ECM expression under different culture conditions. These results suggest that different culture systems are characterized by distinct expression of ECM proteins and their receptors. These results show that it is difficult to extrapolate results from one culture system to another and that cell shape is an important parameter in the cell's ability to respond to the ECM (Watt, 1986). I will therefore attempt to keep most of my references from the literature in this discussion to those studies which are done under anchorage-independent conditions.

7.3 ECM in colony growth

Adhesion to ECM appears (at least superficially) to be the only parameter different between adherent and anchorage-independent cells. It is conceivable that some tumor cells may be able to grow in the complete absence of ECM, however, the cells' ability to respond to ECM proteins and its particular response to ECM may be a key regulator of anchorage-independent growth. Our work, as well as that of others, has shown the presence of ECM proteins in tumor spheroids. Here we provide evidence that ECM proteins are required for growth under anchorage-independent conditions. We have shown that FN is required for SP1 colony growth. Although FN is not sufficient to stimulate colony formation, it appears to play an important role in regulating growth. In support of these results, Coucke *et al* (1992) have shown that preincubation of B16 melanoma cells with FN can increase the formation of lung metastases when injected into mice. In addition, expression of FN is often increased in the stroma of mammary carcinomas (Christensen, 1992). Thus ECM-integrin interactions do play a role in colony formation

in our system and may play a role *in vivo*.

We have also investigated the role of FN fibrils in SP1 colony growth. Inhibition of FN fibril formation with a 70 kDa fragment had no effect on FN-dependent survival but did reduce colony growth. It appears that FN (or collagen) fibril assembly may provide a physical structure for cells, which permit changes in shape and result in different responses to growth factors. In support of this statement, Re *et al* (1994) observed that integrin ligation was not sufficient to inhibit apoptosis of human endothelial cells. Soluble VN could not inhibit apoptosis of these cells, they required to undergo a minimal degree of shape change in order to survive. Tumor cells may have escaped the requirements for cell shape to survive but still require some shape changes to proliferate. These changes are provided by the matrix fibrils or interactions with other cells in the tumor spheroid. We have shown that Coll I can support anchorage-independent growth of Cl-12-H cells. We have yet to investigate whether collagen type I is assembled in fibrils in SP1 colonies. Collagen type I fibril assembly is an entropic process, while FN fibril assembly requires cell surface receptors. Thus the mechanisms by which FN or Coll I fibrils support survival and growth may differ.

An exogenous source of ECM proteins may be most critical at the early stages of colony formation. We have shown that FITC-labelled FN appears to be predominantly located in the central region of the colony. Once the colony has started and matrix fibrils are established, the cells may change their environment by secreting their own ECM proteins and growth factors such as TGF-β which may, in turn, affect ECM production of surrounding cells. The cells would then be less dependent on exogenous factors. Further experiments are required to determine the kinetics for matrix requirements. Removal of

exogenous ECM protein at various time points and following the kinetics of colony growth would establish whether exogenous ECM proteins are required in the early stages of colony formation.

In conclusion, tumor cells can respond to ECM proteins under anchorage-independent conditions; they simply do not spread. SP1 cells can synthesize their own ECM and growth factors under adherent conditions but still require exogenous factors under anchorage-independent conditions, suggesting a shift from autocrine to paracrine mechanisms under anchorage-independent conditions. Once the colony is established, and FN fibril interactions occur, there may be another shift back from paracrine to autocrine growth factor requirements. These data suggest that tumor cells are vulnerable as single cells since they have a high dependence on exogenous factors for survival. In support of this statement, few cells which disseminate from the primary tumor actually go on to form secondary metastases (Weiss *et al.*, 1982). Figure 29 displays a scenario of how ECM and growth factors are involved when an adherent cell becomes anchorage-independent and forms a tumor spheroid.

Figure 29. Hypothetical model for the role of growth factors and ECM in a stepwise progression for colony growth.

Adherent transformed cells progressively lose the requirement for adhesion. Once in an anchorage-independent state, soluble extracellular matrix proteins (ECM) bind to integrin receptors on the cell surface (❨ ❩) and growth factors (GF) bind to their respective receptors (Y) on the cell surface. These interactions provide survival signals to the cell. The cell then can slowly start to proliferate and organize ECM on its cell surface. Once several cells have clustered together, there is formation of ECM fibrils and a change in cell shape which provides the adhesive signals required to proliferate more rapidly and form a three-dimensional spheroid.

Figure 29

Stepwise progression for colony formation

Adherent transformed
cell

ECM, survival, proliferation

Loss of
adhesion
requirement

ECM · GF Survival factors
required

Slow
Proliferation

Cell shape changes

GF GF

Matrix fibrils
provide structure

AI growth
rapid proliferation

GF ECM

Tumor spheroid

GF
?

ECM
?

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7.4 Integrins in anchorage-independent growth

Howlett *et al.* (1995) have suggested that in mammary carcinomas, the interactions between ECM and integrins are disturbed. The right integrins are expressed on the cell surface but the correct differentiation signals are not generated from them. Howlett *et al.* (1995) have shown that Matrigel promoted acinar structure formation by normal mammary cells could be inhibited with anti-β_1 integrin antibodies, while mammary carcinoma cells formed disorganized spheroids in Matrigel which could not be inhibited by anti-β_1 antibodies. They suggested that growth of mammary tumor spheroids was integrin-independent. In contrast, Matrigel increased the metastatic ability of mammary carcinoma cell lines when co-injected with carcinoma cells subcutaneously into nude mice (Mullen *et al.*, 1996). These two results appear to be in conflict with each other. One suggests that tumor cell colony growth is independent of the ECM-integrin interactions while the other suggests that ECM promotes metastasis hence the metastatic process is ECM-dependent. Close analysis of the results by Howlett *et al.* (1995) shows that addition of the anti-β_1 antibodies caused a slight increase in growth of the tumor cells instead of inhibition as seen in normal cells. It is possible that the anti-β_1 antibody is inhibitory in normal cells because they do not recognize the antibody as their natural ligand. In contrast the integrins in transformed cells may not need the specificity provided by the natural ligand and are stimulated by the anti-β_1 antibody as a result of changes in the activation state of the integrin. These changes in activation state (discussed in the Introduction) are likely caused by changes in the activity of cytoplasmic signaling proteins, membrane proteins which alter integrin function or changes in phospholipid composition of the plasma membrane. This is further supported by Werb *et al.* (1989) who demonstrated that synovial fibroblasts

adherent to anti-β_1 antibodies or fragments of FN secreted the proteases stromelysin and collagenase while they did not on intact FN. In some cases an intact ECM molecule may ligate several integrin molecules simultaneously while ECM fragments ligate only one, thereby generating a different signal. These results suggest that parts of the ECM protein are activating while other parts may be suppressive. It was later found that signaling through the $\alpha_5\beta_1$ receptor which recognizes the 120 kDa fragment of FN, increased metalloproteinase expression, while signaling through the $\alpha_4\beta_1$ receptor, which recognizes a different part of FN (CS-1 region) suppressed expression (Huhtala *et al.*, 1995). In addition, proteolytic cleavage of Coll I exposes a cryptic RGD sequence to the $\alpha_v\beta_3$ integrin which is not available on native Coll (Montgomery *et al.*, 1994). It is also known that the ability of integrins to recognize their ligands is regulated by delicate alterations in the conformation of the molecule regulated by divalent cations and intracellular proteins (reviewed in Mould, 1996). In addition, the response of an integrin to an ECM protein may be a result of other factors such as ECM proteins or growth factors which are present in the environment. Together, these results support Howlett *et al* (1995) theory that integrins on transformed cells do not properly recognize their ligands as they showed with human mammary cells.

Although our interest was in the $\alpha_5\beta_1$- fibronectin system on SP1 colony growth, we obtained several results which suggested a role of the $\alpha_2\beta_1$- collagen receptor system in colony growth. (1) The $\alpha_2\beta_1$ integrin expression was increased on the cell surface under anchorage-independent conditions. (2) SP1 cells under anchorage-independent conditions treated with TGF-β only increase their adhesion to collagen (3) SP1 cells which are selected for their anchorage-independent growth properties expressed increased $\alpha_2\beta_1$

expression and collagen could promote their growth in agar.

Tumor cells typically maintain the normal integrin profile but show a reduced or disorganized expression of integrins (Alford and Taylor-Papadimitriou, 1996; Berdichevsky *et al.*, 1994). *De novo* expression or complete loss of integrin expression are seen occasionally in transformed cells. Integrin expression is important for mammary cell function. For example, the $\alpha_2\beta_1$ integrin is required for mammary cell morphogenesis in a collagen gel, while the $\alpha_3\beta_1$ integrin is required for morphogenesis in a laminin rich matrix (Howlett *et al.*, 1995). These data show that different integrins are capable of mediating mammary cell morphogenesis depending on the ECM protein present in the extracellular environment. Saelman *et al* (1995) have demonstrated that loss of $\alpha_2\beta_1$ integrin reduces the ability of MDCK cells to survive and form cysts in three-dimensional collagen gels. The cells lacking $\alpha_2\beta_1$ also demonstrated reduced tubulogenesis and branching morphogenesis in response to HGF. This result also shows a cooperative effect between integrins and growth factors under anchorage-independent conditions.

These results of Howlett *et al* (1995) suggest that it is not a particular integrin receptor which is responsible for increased anchorage-independent growth, but likely several receptors are capable of supporting growth providing that the appropriate ligand is present. Our results support the idea that multiple integrins are involved, since both $\alpha_5\beta_1$ or $\alpha_2\beta_1$ could support anchorage-independent growth in Cl-12-H cells. Cl-12-H cells which express higher levels of $\alpha_2\beta_1$ can respond to Coll I while SP1 and Cl-24-L cells which express lower levels of this receptor do not increase colony growth in response to Coll. Again it appears that the level of receptor expression is an important parameter in determining its function. In support of this observation, overexpression of $\alpha_2\beta_1$ in

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mammary cells tends to restore the normal phenotype. However it is difficult to asses exactly how many receptors are required especially considering that other factors in the environment and proteins on the cell surface may contribute to the response.

7.5 <u>Growth factors and anchorage-independent growth</u>

TGF-β can increase ECM and integrin expression in a number of cell lines and can negatively regulate growth by stimulating cyclin dependent kinase inhibitors (Polyak *et al.*, 1994; Reynisdóttir *et al.*, 1995). TGF-β often has an opposing effect on growth in transformed cells (Welch *et al.*, 1990). It is possible that TGF-β regulates anchorage-independent growth by one of three mechanisms (1) by altering the ECM and integrin expression (2) direct signaling through the receptor affecting cyclins and cdk's or (3) a combination of both. TGF-β can inhibit cell growth of adherent SP1 cells but promotes anchorage-independent growth of these cells in agar. More than just signaling from integrins and TGF-β receptors is required for TGF-β-mediated anchorage-independent growth since FN cannot replace the adhesive signal in suspension. A combination of FN and TGF-β show an additive effect on SP1 colony growth. Cell shape and other parameters mediated by cell adhesion may also contribute to the effects of TGF-β on cell growth (Sutton *et al.*, 1991). Roskelley *et al* (1994) have shown that ECM proteins (Matrigel) are required for milk production in mammary epithelial cells maintained in a rounded morphology on polyHEMA. Adherent cells were much less efficient at expressing β-casein. The effects of individual matrix components or the use of transformed cells were not determined in this model. It is also unclear to what extent a single matrix protein can contribute compared to several components as seen in

experiments with Matrigel.

HGF is a pleiotropic factor reported to affect many cellular functions (see Introduction). It is reported not to affect ECM or integrin expression, however the cellular response to HGF is different depending of the ECM present (Taipale and Keski-Oja, 1996). HGF is also an important regulator of mammary cell growth and morphogenesis. Taking into consideration the last two points, a change in ECM may lead to a change in HGF response and cause a change from HGF-mediated mammary cell morphogenesis to HGF-mediated cell growth. Mammary tumors often express elevated levels of FN and Coll I in the stroma, hence these changes may be accompanied by changes in HGF-mediated signaling. It remains to be determined exactly what parameters are required to regulate HGF-mediated morphogenesis versus growth in mammary cell culture models.

Normal cells in suspension fail to undergo DNA synthesis in the presence of growth factors but show increased expression of c-Myc (Barrett *et al.*, 1995). It was shown that cyclin A production is required for progression through the cell cycle and that fibroblasts in suspension fail to increase cyclin A expression, therefore cyclin A is adhesion dependent. In contrast, Cyclin D and E are more growth factor dependent. Interestingly, TGF-β can override the adhesion requirements in some fibroblasts (Han *et al.*, 1993). One can see a cooperative effect between growth factors and cell adhesion at the level of the cyclins. We have also seen that c-Src, FAK, Ras and PI 3-kinase can function in both integrin and growth factor signaling pathways, however there still remain many unanswered questions regarding the mechanisms by which growth factor and integrin signaling pathways interact.

7.6 Signaling in anchorage-independent growth

One issue which remains to be determined is the specificity of protein-protein interactions after integrin-dependent activation of signal transduction pathways. For example FAK can bind to more than 9 substrates including PI 3-kinase, Src, Csk, HGF receptor, talin, paxillin, p130^{cas}, Grb2 and ß$_1$ integrin. It is likely that FAK does not bind to all these substrates every time it is activated. It may also have different affinities for each substrate. Therefore which substrate is activated will depend on the abundance of FAK, its ligand and its affinity to the particular substrate. When this protein is over-expressed by transfection or naturally through transcriptional regulation, there is a sufficient amount of FAK for both high and low affinity substrates to interact with FAK. This may activate signaling pathways which may not usually be activated under these conditions. Such a mechanism is more clearly seen with integrin expression, where upregulation and downregulation of integrins at the cell surface change the function of the cell. The observation that transfection of the α_5 integrin in CHO cells reduces the transformed phenotype suggests two possible mechanisms: (1) The number of receptors on the cell surface may play an important role in which signals are generated and (2) the signals generated from the integrins are dependent on other signals, hence opposing signals can be generated from the same receptor depending on which other signaling pathways or molecules are active. In support of this evidence, Varner *et al* (1995) demonstrated that unligated receptor generated negative signals for growth while ligated receptor generated positive signals for growth. There is also increasing evidence that there are cooperative interactions between different integrin receptors and between growth factors and integrin signaling pathways. In contrast we show that high expression of $\alpha_5\beta_1$ promotes colony growth.

However it may be that other parameters are also involved or the level of expression of $\alpha_5\beta_1$ in our cells is still not as high as compared to transfected cells. The absolute number of receptors per cell would have to be calculated. It would be interesting to compare various integrin signaling mechanisms based on the level of integrin expression on the cell surface. This could be achieved by using the tetracycline inducible promoter in which expression can be regulated by the concentration of tetracycline added.

7.7 In conclusion

The normal differentiated phenotype is maintained by a complex balance of ECM and growth factors with the appropriate kind and number of cell surface receptors. Once a cell becomes transformed a change in any one of the components may cause a chain reaction of events which, with time, permit a progressive fully transformed cell. ECM and adhesion can modulate growth factor expression and vice-versa. *In vivo*, the balance can not restore itself but there are several experimental models where transformed cells are forced to show a differentiated phenotype by modulating their environment or restoring the balance of cell surface receptors.

Transformation is a stepwise process which occurs after many mutations. Cells are therefore becoming gradually transformed. At early stages, ECM-integrin interactions may promote differentiation while at later stages, promote migration and invasion during progression to the transformed and metastatic phenotype.

In conclusion, normal and transformed cells respond to the ECM under non-adherent conditions but it is the interpretation of the cellular signals generated as a result of ECM-integrin interactions which decides whether the cell will differentiate or proliferate. These signals depend on a number of factors (1) degree of differentiation of the cells, (2) the

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quality and quantity of cell surface receptors, (3) the presence and type of growth factors, (4) cell type and (5) cell shape. With a full understanding of all the parameters it would seem possible that transformed cells with different degrees of malignancy can be stimulated to redifferentiate providing the correct environment is provided.

7.8 The big picture

One has to be cautious in extrapolating results obtained *in vitro* to an *in vivo* situation since there are many other factors present *in vivo* which affect tumor cell function and phenotype. Here I describe an *in vivo* metastatic cascade and describe at what stages the findings of this thesis may contribute.

Tumor cells grow at the site of origin until the tumor becomes vascularized, then the tumor cells escape into the circulation. Once in the circulation, the cells must survive under anchorage-independent conditions and extravasate at a distant site and grow into a secondary tumor or metastasis. The properties of metastatic cells are therefore different from the primary tumor cells.

This thesis makes several contributions on the understanding of this process. Once cells escape from the primary tumor, and enter the circulation, factors in the serum such as ECM proteins or growth factors act as survival factors for some tumor cells (i.e. few cells can respond to these factors). We have shown that FN, HGF and TGF-β support survival of SP1 cells, however, other serum factors are likely also support this function *in vivo* depending on the specific phenotype of the tumor cell. The increase in the $\alpha_2\beta_1$ integrin (laminin and collagen receptor) would be useful in adhesion to the basement membrane at a distant site which is primary composed of collagen type IV and laminin. Once it has extravasated at a distant site, the tumor may grow slowly at first, but will become vascularized. Since new blood vessels tend to be very leaky, they would provide plenty of

plasma proteins such as FN for tumor growth. For tumor cells which do not express sufficient fibronectin receptors , expression of collagen receptors could substitute and exogenous source of collagen could be provided by the newly formed endothelial cells or surrounding stromal fibroblasts. Our results also demonstrate that survival and anchorage-independent growth are separate steps and that fibril formation is not required for cell survival but is required for colony growth.

This thesis has made some contributions to the understanding of the role of ECM protein in anchorage-independent growth of a murine mammary carcinoma cell line. An understanding of the growth requirements of tumor cells in spheroids will improve our ability to treat metastatic disease, the primary cause of cancer mortality.

BIBLIOGRAPHY

Adams,J.C. and Watt,F.M. Changes in keratinocyte adhesion during terminal differentiation: reduction in fibronectin binding precedes $\alpha_5\beta_1$ integrin loss from the cell surface. Cell. 63:425-435, 1990.

Akiyama,S.K. and Yamada,K.M. The interaction of plasma fibronectin with fibroblasts in cell suspension. J. Biol. Chem. 260:4492-4500, 1985.

Akiyama,S.K., Yamada,S.S., Yamada,K.M. and LaFlamme,S.E. Transmembrane signal transduction by integrin cytoplasmic domains expressed in single-subunit chimeras. J. Biol. Chem. 269:15961-15964, 1994.

Akiyama,S.K., Olden,K. and Yamada,K.M. Fibronectin and integrins in invasion and metastasis. Cancer and Metastasis Rev. 14:173-189, 1995.

Albelda,S.M. and Buck,C.A. Integrins and other cell adhesion molecules. FASEB J. 4:2868-2880, 1990.

Alford,D. and Taylor-Papadimitriou,J. Cell adhesion molecules in the normal and cancerous mammary gland. J. Mammary Gland Biol. Neo. 1:207-218, 1996.

Alitalo,K. and Vaheri,A. Pericellular matrix in malignant transformation. Adv. Cancer. Res. 37: 111-158, 1982.

Aota,S., Nomizu,M. and Yamada,K.M. The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function. J. Biol. Chem. 269:24756-24761, 1994.

D'Ardenne,A.J., Richman, P.I., Horton, M.A., Mcaulay, A.E. and Jordan,S. Co-ordinate expression of the alpha6 integrin laminin receptor sub-unit and laminin in breast cancer. J. Pathol. 165:213-220, 1991.

Barrett,J.F., Lewis,B.C., Hoang,A.T., Alvarez,R.J. Jr, and Dang,C.V. Cyclin A links c-myc to adhesion-independent cell proliferation. J. Biol. Chem. 270:15923-15925, 1995.

Bauer,J.S., Schreiner,C.L., Giancotti,F.G., Ruoslahti,E. and Juliano,R.L. Motility of fibronectin receptor-deficient cells on fibronectin and vitronectin: collaborative interactions among integrins. J. Cell Biol. 116:477-487, 1992.

Bauer,J.S., Varner,J., Schreiner,C., Kornberg,L., Nicholas,R. and Juliano,R.L. Functional role of the cytoplasmic domain of the integrin α_5 subunit. J. Cell Biol. 122:209-221, 1993.

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Belkin,A.M., Zhidkova,N.I., Balzac,F., Altruda,F., Tomatis,D., Maier,A., Tarone,G., Koteliansky,V.E. and Burridge,K. β_{1D} integrin displaces the β_{1A} isoform in striated muscles: localization at junctional structures and signaling potential in nonmuscle cells. J. Cell Biol. 132:211-226, 1996.

Berdichevsky,F., Gilbert,C., Shearer,M. and Taylor-Papadimitriou,J. Collagen-induced rapid morphogenesis of human mammary epithelial cells: the role of the $\alpha_2\beta_1$ integrin. J. Cell Sci. 102:437-446, 1992.

Berdichevsky,F., Wetzels,R., Shearer,M., Martignone,S., Ramaekers,F.C.S. and Taylor-Papadimitriou,J. Integrin expression in relation to cell phenotype and malignant change in the human breast. Mol. Cell. Differ. 2:255-274, 1994.

Blystone,S.D., Graham,I.L., Lindberg,F.P., and Brown,E. Integrin $\alpha_v\beta_3$ differentially regulates adhesive and phagocytic functions of the fibronectin receptor $\alpha_5\beta_1$. J. Cell. Biol. 127:1129-1137, 1994.

Bojanowski,K., Lelievre,S., Markovits,J., Couprie,J., Jacquemin-Sablon,A.J. and Larsen,A.K. Suramin is an inhibitor of DNA topoisomerase II *in vitro* and in chinese hamster fibrosarcoma cells. Proc. Natl. Acad. Sci. USA. 89:3025-3029, 1992.

Boudreau,N., Sympson,C.J., Werb,Z. and Bissell,M.J. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science. 267:891-893, 1995.

Boudreau,N., Werb,Z., and Bissell,M.J. Suppression of apoptosis by basement membrane requires three-dimensional tissue organization and withdrawal from the cell cycle. Proc. Natl. Acad. Sci. USA. 93:3509-3513, 1996.

Bruijn,J.A. and de Heer,E. Adhesion molecules in renal diseases. Lab. Invest. 72:387-394, 1995.

Byers,S., Park,M., Sommers,C. and Seslar,S. Breast carcinoma: a collective disorder. Breast Cancer Res. Treat. 31:203-215, 1994.

Cannon-Albright,L.A. and Skolnick,M.H. The genetics of familial breast cancer. Sem. Oncol. 23:1-5, 1996.

Carpenter,C.L. and Cantley,L.C. Phosphoinositide 3-kinase and the regulation of cell growth. Biochim. Biophys. Acta. 1288:M11-M16, 1996.

Chammas,R., Veiga,S.S., Travassos,L.R. and Brentani,R.R. Functionally distinct roles for glycosylation of α and β integrin chains in cell-matrix interactions. Proc. Natl. Acad. Sci. USA. 90:1795-1799, 1993.

Chan,B.M.C., Kassner,P.D., Schiro,J.A., Byers,H.R., Kupper,T.S. and Hemler,M.E. Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. Cell. 68:1051-1060, 1992.

Charo,I.F., Nannizzi,L., Smith,J.W. and Cheresh,D.A. The vitronectin receptor $\alpha_v\beta_3$ binds fibronectin and acts in concert with $\alpha_5\beta_1$ in promoting cellular attachment and spreading on fibronectin. J. Cell Biol. 111:2795-2800, 1990.

Chen,D., Magnuson,V., Hill,S., Arnaud,C., Steffensen,B. and Klebe,R.J. Regulation of integrin gene expression by substrate adherence. J Biol. Chem. 267:23502-23506, 1992.

Chen,Q., Kinch,M.S., Lin,T.H., Burridge,K. and Juliano,R.L. Integrin-mediated cell adhesion activates mitogen-activated protein kinase. J. Biol. Chem. 269:26602-26605, 1994.

Christensen,L. The distribution of fibronectin, laminin and tetranectin in human breast cancer with special attention to the extracellular matrix. APMIS. Suppl. 26:6-39, 1992.

Christensen,L., Nielsen,M., Andersen,J. and Clemmensen,I. Stromal fibronectin staining pattern and metastasizing ability of human breast carcinoma. Cancer Res. 48:6227-6233, 1988.

Clark,E.A. and Brugge,J.S. Integrins and signal transduction pathways: the road taken. Science. 268:233-239, 1995.

Clark,P. Modulation of scatter factor/hepatocyte growth factor activity by cell-substratum adhesion. J. Cell. Sci. 107:1265-1275, 1994.

Clarke,A.S., Lotz,M.M., Chao,C. and Mercurio,A.M. Activation of the p21 pathway of growth arrest and apoptosis by the β_4 integrin cytoplasmic domain. J. Biol. Chem. 270:22673-22676, 1995.

Coffey,R.J. Jr., Leof,E.B., Shipley,G.D. and Moses,H.L. Suramin inhibition of growth factor receptor binding and mitogenicity in AKR-2B cells. J. Cell. Physiol. 132:143-148, 1987.

Conforti,G., Zanetti,A., Pasquali-Ronchetti,I., Quaglino,D. Jr., Neyroz,P. and Dejana,E. Modulation of vitronectin receptor binding by membrane lipid composition. J. Biol. Chem. 265:4011:4019, 1990.

Coucke,P., De Pauw-Gillet,M.C., De Leval.L., Noel,A., Bassleer,R. and Foidart,J.M. Fibronectin promotes lung colony formation in the mouse by B16 melanoma cells spheroids. In vivo. 6:481-486, 1992.

Craig,S.W. and Johnson,R.P. Assembly of focal adhesions: progress, paradigms, and portents. Curr.Opin. Cell Biol. 8:74-85, 1996.

Dalton,S.L., Marcantonio,E.E. and Assoian,R.K. Cell attachment controls fibronectin and $\alpha_5\beta_1$ integrin levels in fibroblasts. Implications for anchorage-dependent and - independent growth. J. Biol. Chem. 267:8186-8191, 1992.

Dean,D.C., Newby,R.F. and Bourgeois,S. Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor β, and cAMP in human cell lines. J. Cell Biol. 106:2159-2170, 1988.

Dedhar,S. Integrins and tumor invasion. Bioassays. 12:1-8, 1990.

Dedhar,S. and Hannigan,G.E. Integrin cytoplasmic interactions and bidirectional transmembrane signalling. Curr. Opin. Cell Biol. 8:657-669, 1996.

Dedhar,S. and Saulnier,R. Alterations in integrin receptor expression on chemically transformed human cells. Specific enhancement of laminin and collagen receptor complexes. J. Cell Biol. 110:481-489, 1990.

Delcommenne,M. and Streuli,C.H. Control of integrin expression by extracellular matrix. J. Biol. Chem. 270:26794-26801, 1995.

Dzamba,B.J., Wu,H., Jaenisch,R. and Peters,D.M. Fibronectin binding site in type I collagen regulates fibronectin fibril formation. J. Cell Biol. 121:1165-1172, 1993.

Elliott,B.E., Maxwell,L., Arnold,M., Wei,W.Z. and Miller,F.R. Expression of epithelial-like markers and class I major histocompatibility antigens by a murine carcinoma growing in the mammary gland and in metastases: Orthotropic site effects. Cancer Res. 48:7237-7245, 1988.

Elliott,B.E., Tam,S.P., Dexter,D. and Chen,Z.Q. Capacity of adipose tissue to promote growth and metastasis of a murine mammary carcinoma: effect of estrogen and progesterone. Int. J. Cancer. 51:416:424, 1992.

Engel,J. Efimov,V.P. and Maurer,P. Domain organizations of extracellular matrix proteins and their evolution. Devel. Suppl. 35-42, 1994.

Fang,F., Orend,G., Watanabe,N., Hunter,T. and Ruoslahti,E. Dependance of cyclin E-cdk2 kinase activity on cell anchorage. Science. 271:499-502,1996.

Fässler,R., Georges-Labouesse,E. and Hirsch,E. Genetic analyses of integrin function in mice. Curr. Opin. Cell Biol. 8:641-646, 1996.

Faull,R.J., Kovach,N.L., Harlan,J.M. and Ginsberg,M.H. Affinity modulation of the

integrin $\alpha_5\beta_1$: regulation of the functional response by soluble fibronectin J. Cell Biol. 121:155-162, 1993.

Fehlner-Gardiner,C.C., Uniyal,S., von Ballestrem,C.G. and Chan,B.M.C. Differential utilization of VLA-4 ($\alpha4\beta1$) and -5 ($\alpha5\beta1$) integrins during the development of mouse bone marrow-derived mast cells. Differentiation. 60:317-325, 1996.

Fields,G.B. Synthetic peptides and tumor cell metastasis. Peptide Res. 6:115-120, 1993.

Fitzpatrick,D.R., Bielefeldt-Ohmann,H., Himbeck,R.P., Jarnicki,A.G., Marzo,A.L. and Robinson,B.W.S. Transforming growth factor-beta: antisense RNA-mediated inhibition affects anchorage-independent growth, tumorigenicity and tumor-infiltrating T-cell in malignant mesothelioma. Growth Factors. 11:29-44, 1994.

Foekens,J.A., Sieuwerts,A.M., Stuurman-Smeets,E.M.J., Peters,H.A. and Klijn,J.G.M. Effects of suramin on cell-cycle kinetics of MCF-7 human breast cancer cells in vitro. Br. J. Cancer 67:232-236, 1993.

Fogerty,F.J., Akiyama,S.K., Yamada,K.M. and Mosher,D.F. Inhibition of binding of fibronectin to matrix assembly sites by anti-integrin ($\alpha_5\beta_1$) antibodies. J. Cell Biol. 111:699-708, 1990.

Folkman,J. and Moscona,A. Role of cell shape in growth control. Nature. 273:345-349, 1978.

Freedman,V.H. and Shin,S.I. Cellular tumorigenicity in nude mice: Correlation with cell growth in semi-solid medium. Cell. 3:355-359,1974.

Frisch,S.M. and Francis,H. Disruption of epithelial cell-matrix interactions induces apoptosis. J. Cell Biol. 124:619-626, 1994.

Frisch,S.M., Vuori,K., Ruoslahti,E. and Chan-Hui,P.Y. Control of adhesion-dependent cell survival by focal adhesion kinase. J. Cell Biol. 134:793-799, 1996.

Galéra,P., Musso,M., Ducy,P. and Karsenty,G. c-Krox, a transcriptional regulator of type 1 collagen gene expression, is preferentially expressed in skin. Proc. Natl. Acad. Sci. USA. 91:9372-9376, 1994.

Giancotti,F.G. and Mainiero,F. Integrin-mediated adhesion and signaling in tumorigenesis. Biochim. Biophys. Acta. 1198:47-64, 1994.

Giancotti,F.G. and Ruoslahti,E. Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of chinese hamster ovary cells. Cell. 60:849-859, 1990.

Glimelius,B., Norling,B., Nederman,T. and Carlsson,J. Extracellular matrices in multicellular spheroids of human glioma origin: increased incorporation of proteoglycans and fibronectin as compared to monolayer cultures. APMIS. 96:433-444, 1988.

Guadagno,T.M. and Assoian,R.K. G1/S control of anchorage-independent growth in the fibroblast cell cycle. J. Cell Biol. 115:1419-1425, 1991.

Guadagno,T.M., Ohtsubo,M., Roberts,J.M. and Assoian,R.K. A link between cyclin A expression and adhesion-dependent cell cycle progression. Science. 262:1572-1575, 1993.

Guan,J.L. and Chen,H.C. Signal transduction in cell-matrix interactions. Int. Rev. Cytology. 168:81-121, 1996.

Guan,J.L. and Hynes,R.O. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha_4\beta_1$. Cell. 60:53-61, 1990.

Gui,G.P.H., Puddefoot,J.R., Vinson,G.P., Wells,C.A. and Carpenter,R. *In vitro* regulation of human breast cancer cell adhesion and invasion via integrin receptors to the extracellular matrix. Br. J. Surg. 82:1192-1196, 1995a.

Gui,G.P.H., Wells,C.A., Browne,P.D., Yeomans,P., Jordan,S., Puddefoot,J.R., Vinson,G.P. and Carpenter,R. Integrin expression in primary breast cancer and its relation to axillary nodal status. Surg. 17:102-108, 1995b.

Han,E.K.H., Guadagno,T.M., Dalton,S.L. and Assoian,R.K. A cell cycle and mutational analysis of anchorage-independent growth: cell adhesion and TGF-β_1 control G1/S transit specifically. J. Cell Biol. 122:461-471, 1993.

Hannigan,G.E., Leung-Hagesteijn,C., Fitz-Gibbon,L., Coppolino,M.G., Radeva,G., Filmus,J., Bell,J.C. and Dedhar,S. Regulation of cell adhesion and anchorage-dependent growth by a new β_1-integrin-linked protein kinase. Nature. 379:91-96, 1996.

Hara,K., Yonezawa,K., Sakaue,H., Ando,A., Kotani,K., Kitamura,T., Kitamura,Y., Ueda,H., Stephens,L., Jackson,T.R., Hawkins,P.T., Dhand,R., Clark,A.E., Holman,G.D., Waterfield,M.D. and Kasuga,M. 1-Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells. Proc. Natl. Acad. Sci. USA. 91:7415-7419, 1994.

Hauptmann,S., Denkert,C., Löhrke,H., Tietze,L., Ott,S., Klosterhalfen,B. and Mittermayer,C. Integrin expression on colorectal tumor cells growing as monolayers, as multicellular tumor spheroids, or in nude mice. Int. J. Cancer. 61:819-825, 1995.

Heino,J., Ignotz,R.A., Hemler,M.A., Crouse,C. and Massagué,J. Regulation of cell adhesion receptors by transforming growth factor-β. Concominant regulation of integrins that share a common β_1 subunit. J. Biol. Chem. 265:380-388, 1989.

Hocking,D.C., Sottile,J. and McKeown-Longo,P.J. Fibronectin's III-1 module contains a conformation-dependent binding site for the amino-terminal region of fibronectin. J Biol. Chem. 269:19183-19187, 1994.

Hogervorst,F., Kuikman,I., Kr von dem Borne,A.E.G. and Sonnenberg,A. Cloning and sequence analysis of beta-4 cDNA: an integrin subunit that contains a unique 118kd cytoplasmic domain. EMBO J. 9: 765-770, 1990.

Hotchin,N.A., Kovach,N.L. and Watt,F.M. Functional down-regulation of $\alpha_5\beta_1$ integrin in keratinocytes is reversible but commitment to terminal differentiation is not. J. Cell Sci. 106:1131-1138, 1993.

Howeedy,A.A., Virtanen,I., Laitinen,L., Gould,N.S., Koukoulis,G.K. and Gould,V.E. Differential distribution of tenascin in the normal, hyperplastic, and neoplastic breast. Lab. Invest. 63:789-806, 1990.

Howlett,A.R., Bailey,N., Damsky,C.H., Petersen,D.W. and Bissell,M.J. Cellular growth and survival are mediated by β_1 integrins in normal human breast epithelium but not in breast carcinoma. J. Cell Sci. 108:1945-1957, 1995.

Howlett,A.R. and Bissell,M.J. The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary epithelium. Epith. Cell. Biol. 2:79-89, 1993.

Hu,D.D., Hoyer,J.R. and Smith,J.W. Ca^{2+} suppresses cell adhesion to osteopontin by attenuating binding affinity for integrin $\alpha_v\beta_3$. J. Biol. Chem. 270:9917-9925, 1995.

Hudson,B.G., Reeders,S.T. and Tryggvason,K. Type IV collagen; structure, gene organization and role in human diseases. J. Biol. Chem. 268:26033-26036, 1993.

Huhtala,P., Humphries,M.J., McCarthy,J.B., Tremble,P.M., Werb,Z. and Damsky,C.H. Cooperative signaling by $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. J. Cell Biol. 129:867-879, 1995.

Hynes,R.O. Integrins: A family of cell surface receptors. Cell. 48: 549-554, 1987.

Isberg,R.R. and Leong,J.M. Multiple β_1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell. 60: 861-871, 1990.

Jiang,W.G., Lloyds,D., Puntis,M.C.A., Nakamura,T. and Hallett,M.B. Regulation of spreading and growth of colon cancer cells by hepatocyte growth factor. Clin. Exp. Metastasis 11:235-242, 1993.

Jockusch,B.M., Bubeck,P., Giehl,K., Kroemker,M., Moschner,J., Rothkegel,M., Rüdiger,M., Schlüter,K., Stanke,G. and Winkler,J. The molecular architecture of focal adhesions. Ann. Rev.Cell Dev. Biol. 11:379-416, 1995.

Johansson,S. Demonstration of high affinity fibronectin receptors on rat hepatocytes in suspension. J. Biol. Chem. 260:1557-1561, 1985.

Juliano,R.L. and Varner,J.A. Adhesion molecules in cancer: the role of integrins. Curr. Opin. Cell Biol. 5:812-818, 1993.

Kadler,K.E., Holmes,D.F., Trotter,J.A. and Chapman,J.A. Collagen fibril formation. Biochem. J. 316:1-11, 1996.

Kanda,H., Tajima,H., Lee,G.H., Nomura,K., Ohtake,K., Matsumoto,K., Nakamura,T. and Kitagawa,T. Hepatocyte growth factor transforms immortalized mouse liver epithelial cells. Oncogene. 8:3047-3053, 1993.

Kang,J.S. and Krauss,R.S. Ras induces anchorage-independent growth by subverting multiple adhesion-regulated cell cycle events. Mol. Cell. Biol. 16:3370-3380, 1996.

Kaplan,K.B., Swedlow,J.R., Morgan.D.D. and Varmus,H.E. C-Src enhances the spreading of src$^{-/-}$ fibroblasts on fibronectin by a kinase-independent mechanism. Genes Devel. 9:1505-1517, 1995.

Kapron-Bras,C., Fitz-Gibbon,L., Jeevaratnam,P., Wilkins,J. and Dedhar,S. Stimulation of tyrosine phosphorylation and accumulation of GTP bound p21^{ras} upon antibody-mediated $\alpha_2\beta_1$ integrin activation in T-lymphoblastic cells. J. Biol. Chem. 268:20701-20704, 1993.

Kato,M., Saunders,S., Nguyen,H. and Bernfield,M. Loss of cell surface syndecan-1 causes epithelia to transform into anchorage-independent mesenchyme-like cells. Mol. Biol. Cell. 6:559-576, 1995.

Kawano,T., Takasaki,S., Tao,T.W. and Kobata,A. Altered glycosylation of β_1 integrins associated with reduced adhesiveness to fibronectin and laminin. Int. J. Cancer. 53:91-96, 1993.

Kemperman,H., Wijnands,Y., Meijne,A.M.L. and Roos,E. TA3/st, but not TA3/Ha, mammary carcinoma cell adhesion to hepatocytes is mediated by $\alpha_5\beta_1$ interacting with surface-associated fibronectin. Cell Adhesion Comm. 2:45-58, 1994.

Kim,K., Akoto-Amanfu,E., Cherrick,H.M. and Park,N.H. Anchorage-independent growth and the expression of cellular proto-oncogenes in normal human epidermal keratinocytes and in human squamous cell carcinoma cell lines. Oral Surg. Oral Med. Oral Path. 71:303-311, 1991.

Kirchhofer,D., Languino,L.R., Ruoslahti,E. and Pierschbacher,M.D. $\alpha_2\beta_1$ integrins from different cell types show different binding specificities. J. Biol. Chem. 265:615-618, 1990.

Kishimoto,T.K., Jutila,M.A., Berg,E.L. and Butcher,E.C. Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. Science. 245:1238-1241, 1989.

Klein,C.E., Dressel,D., Steinmayer,T., Mauch,C., Eckes,B., Krieg,T., Bankert,R.B. and Weber,L. Integrin $\alpha_2\beta_1$ is upregulated in fibroblasts and highly aggressive melanoma cells in three-dimensional collagen lattices and mediates the reorganization of collagen I fibrils. J. Cell Biol. 115:1427-1436, 1991.

Kleinman,H.K., McGarvey,M.L., Hassell,J.R., Star,V.L., Cannon,F.B., Laurie,G.W. and Martin,G.R. Basement membrane complexes with biological activities. Biochem. 25:312-318, 1986.

Koli,K. and Keski-Oja,J. Transforming growth factor-β system and its regulation by members of the steroid-thyroid hormone superfamily. Adv. Cancer Res. 70:63-94, 1996.

Koukoulis,G.K., Virtanen,I., Korhonen,M., Laitinen,L., Quaranta,V. and Gould,V.E. Immunohistochemical localization of integrins in the normal, hyperplastic, and neoplastic breast. Am. J. Pathol. 139:787-799, 1991.

Kuzumaki,T., Matsuda,A., Ito,K. and Ishikawa,K. Cell adhesion to substratum and activation of tyrosine kinases are essentially required for G1/S phase transition in BALB/c 3T3 fibroblasts. Biochim. Biophys. Acta. 1310:185-192, 1996.

Lacovara,J., Cramer,E.B., and Quigley,J.P. Fibronectin enhancement of directed migration of B16 melanoma cells. Cancer Res. 44:1657-1663, 1984.

Lafrenie,R.M. and Yamada,K.M. Integrin-dependent signal transduction. J. Cell. Biochem. 61:543-553, 1996.

Lampe,B.V., Stallmach,A. and Riecken,E.O. Altered glycosylation of integrin adhesion molecules in colorectal cancer cells and decreased adhesion to the extracellular matrix. Gut. 34:829-836, 1993.

Lamszus,K., Joseph,A., Jin,L., Yao,Y., Chowdhury,S., Fuchs,A., Polverini,P.J., Goldberg,I.D. and Rosen,E.M. Scatter factor binds to thrombospondin and other extracellular matrix components. Am. J. Pathol. 149:805-819, 1996.

Languino,L.R., Gehlsen,K.R., Wayner,E., Carter,W.G., Engvall,E. and Ruoslahti,E. Endothelial cells use $\alpha_2\beta_1$ integrin as a laminin receptor. J. Cell. Biol. 109:2455-2462, 1989.

Larsen,A.K. Suramin: an anticancer drug with unique biological effects. Cancer Chemother. Pharm. 32:96-98, 1993.

Larson,R.S., Hibbs,M.L. and Springer,T.A. The leukocyte integrin LFA-1 reconstituted by cDNA transfection in a nonhematopoietic cell line is functionally active and not transiently regulated. Cell Reg. 1:359-367, 1990.

Larson,R.S. and Springer,T.A. Structure and function of leukocyte integrin. Immunol. Rev. 114:181-217, 1990.

Laurie,G.W., Leblond,C.P. and Martin,G.R. Localization of type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin to the basal lamina of basement membranes. J Cell Biol. 95:340-344, 1982.

Leppä,S., Mali,M., Miettinen,H.M. and Jalkanen,M. Syndecan expression regulates cell morphology and growth of mouse mammary epithelial tumor cells. Proc. Natl. Acad. Sci. USA. 89:932-936, 1992.

Lester,B.R. and McCarthy,J.B. Tumor cell adhesion to the extracellular matrix and signal transduction mechanisms implicated in tumor cell motility, invasion and metastasis. Cancer Metastases Rev. 11:31-44, 1992.

Lin,M.L. and Bertics,P.J. Laminin responsiveness is associated with changes in fibroblast morphology, motility, and anchorage-independent growth: cell system for examining the interaction between laminin and EGF signaling pathways. J. Cell. Physiol. 164:593-604, 1995.

Lochter,A. and Bissell, M.J. Involvement of extracellular matrix constituents in breast cancer. Sem. Cancer Biol. 6:165-173, 1995.

Maemura,M., Akiyama,S.K., Woods Virgil,L.Jr. and Dickson,R.B. Expression and ligand binding of $\alpha_2\beta_1$ integrin on breast carcinoma cells. Clin. Exp. Metastasis. 13:223-235, 1995.

Mainiero,F., Pepe,A., Wary,K.K., Spinardi,L., Mohammadi,M., Schlessinger,J. and Giancotti,F.G. Signal transduction by the $\alpha_6\beta_4$ integrin: distinct β_4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. EMBO J. 14:4470-4481, 1995.

Marcantonio,E.E., Guan,J.L., Trevitnick,J.E. and Hynes,R.O. Mapping of the functional determinants of the β_1 cytoplasmic domain by site-directed mutagenesis. Cell Regul. 1:597-604, 1990.

Mardon,H.J., Grant,R.P., Grant,K.E. and Harris,H. Fibronectin splice variants are differentially incorporated in the extracellular matrix of tumorigenic and non-tumorigenic hybrids between normal fibroblasts and sarcoma cells. J. Cell Sci. 104:783-792, 1993.

Marshall,J.F., Rutherford,D.C., McCartney,A.C.E., Mitjans,F., Goodman,S.L. and Hart,I.R. $\alpha_v\beta_1$ is a receptor for vitronectin and fibrinogen, and acts with $\alpha_5\beta_1$ to mediate spreading on fibronectin. J. Cell Sci. 108:1227-1238, 1995.

Masuda,A., Kizaka-Kondoh,S., Miwatani,H., Terada,Y., Nojima,H. and Okayama,H. Signal transduction cascade shared by epidermal growth factor and platelet-derived growth factor is a major pathway for oncogenic transformation in NRK cells. New Biol. 4:489-503, 1992.

Matsumoto,K., Matsumoto,K., Nakamura,T. and Kramer,R.H. Hepatocyte growth factor/scatter factor induces tyrosine phosphorylation of focal adhesion kinase (p125^{FAK}) and promotes migration and invasion by oral squamous cell carcinoma cells. J. Biol. Chem. 269:31807-31813, 1994.

Matsumoto,K., Kagoshima,M. and Nakamura,T. Hepatocyte growth factor as a potent survival factor for rat pheochromocytoma PC12 cells. Exp. Cell Res. 220:71-78, 1995a.

Matsumoto,K., Ziober,B.L., Yao,C.C. and Kramer,R.H. Growth factor regulation of integrin-mediated cell motility. Cancer Metastasis Rev. 14:205-217, 1995b.

McKeown-Longo,P.J. and Mosher,D.F. Interaction of the 70,000-mol-wt amino-terminal fragment of fibronectin with the matrix-assembly receptor of fibroblasts. J. Cell Biol. 100:364-374, 1985.

McNamee,H.P., Ingber,D.E. and Schwartz,M.A. Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. J. Cell Biol. 121:673-678, 1993.

Meredith,J.E., Fazeli,B. and Schwartz,M.A. The extracellular matrix as a cell survival factor. Mol. Biol. Cell. 4:953-961, 1993.

Meredith,J.E.Jr., Takada,Y., Fornaro,M., Languino,L.R. and Schwartz,M.A. Inhibition of cell cycle progression by the alternatively spliced integrin β_{1C}. Science. 269:1570-1572, 1995.

Miyamoto,S., Akiyama,S.K. and Yamada,K.M. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. Science. 267:883-885, 1995a.

Miyamoto,S., Teramoto,H., Coso,O.A., Gutkind,J.S., Burbelo,P.D., Akiyama,S.K. and Yamada,K.M. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J. Cell Biol. 131:791-805, 1995b.

Montgomery,A.M.P., Reisfeld,R.A. and Cheresh,D.A. Integrin $\alpha_v\beta_3$ rescues melanoma cells from apoptosis in three-dimensional dermal collagen. Proc. Natl. Acad. Sci. USA. 91:8856-8860, 1994.

Moon,K.Y., Shin,K.S., Song,W.K., Chung,C.H., Ha,D.B. and Kang,M.S. A candidate molecule for the matrix assembly receptor to the N-terminal 29-kDa fragment of fibronectin in chick myoblasts. J. Biol. Chem. 269:7651-7657, 1994.

Mortarini,R., Gismondi,A., Santoni,A., Parmiani,G. and Anichini,A. Role of the $\alpha_5\beta_1$ integrin receptor in the proliferative response of quiescent human melanoma cells to fibronectin. Cancer Res. 52:4499-4506, 1992.

Mould,P.A. Getting integrins into shape: recent insights into how integrin activity is regulated by conformational changes. J. Cell Sci. 109:2613-2618, 1996.

Mould,P.A., Akiyama,S.K. and Humphries,M.J. Regulation of integrin $\alpha_5\beta_1$-fibronectin interactions by divalent cations. Evidence for distinct classes of binding sites for Mn^{2+}, Mg^{2+}, and Ca^{2+}. J. Biol. Chem. 270: 26270-26277, 1995.

Mullen,P., Ritchie,A., Langdon,S.P. and Miller,W.R. Effect of matrigel on the tumorigenicity of human breast and ovarian carcinoma cell lines. Int. J. Cancer. 67:816-820, 1996.

Murata,J., Saiki,I., Yoneda,J. and Azuma,I. Differences in chemotaxis to fibronectin in weakly and highly metastatic tumor cells. Jpn. J. Cancer Res. 83:1327-1333, 1992.

Nederman,T., Norling,B., Glimelius,B., Carlsson,J. and Brunk,U. Demonstration of an extracellular matrix in multicellular tumor spheroids. Cancer Res. 44: 3090-3097, 1984.

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Newman,M.J., Lane,E.A., Nugent,M.A. and Racker,E. Induction of anchorage-independent growth by epidermal growth factor and altered sensitivity to type β transforming growth factor in partially transformed rat kidney cells. Cancer Res. 46:5842-5850, 1986.

Niranjan,B., Buluwela,L., Yant,J., Perusinghe,N., Atherton,A., Phippard,D., Dale,T., Gusterson,B. and Kamalati,T. HGF/SF: a potent cytokine for mammary growth, morphogenesis and development. Devel. 121:2897-2908, 1995.

Numa,F., Hirabayashi,K., Tsunaga,N., Kato,H., O'Rourke,K., Shao,H., Stechmann-Lebakken,C., Varani,J., Rapraeger,A. and Dixit,V.M. Elevated levels of syndecan-1 expression confer potent serum-dependent growth in human 293T cells. Cancer Res. 55:4676-4680, 1995.

Oda,K., Hori,S., Itoh,H., Osamura,Y., Tokuda,Y., Kubota,M. and Tajima,T. Immunohistochemical study of transforming growth factor-β, fibronectin and fibronectin receptor in invasive mammary carcinomas. Acta. Pathol. Jpn. 42:645-650, 1992.

Olive,P.L. and Durand,R.E. Drug and radiation resistance in spheroids: cell contact and kinetics. Cancer Metastasis Rev. 13:121-138, 1994.

Ono,K., Nakane,H. and Fukushima,M. Differential inhibition of various deoxyribonucleic and ribonucleic acid polymerases by suramin. Eur. J. Biochem. 172:349-353, 1988.

Otey,C.A., Pavalko,F.M. and Burridge,K. An interaction between α-actinin and the β_1 integrin subunit *in vitro*. J. Cell Biol. 111:721-729, 1990.

Owens,L.V., Xu,L., Craven,R.J., Dent,G.A., Weiner,T.M, Kornberg,L., Liu,E.T. and Cance,W.G. Overexpression of the focal adhesion kinase (p125[FAK]) in invasive human tumors. Cancer Res. 55:2752-2755, 1995.

Oyama,F., Hirohashi,S., Sakamoto,M., Titani,K. and Sekiguchi,K. Coordinate oncodevelopmental modulation of alternative splicing of fibronectin pre-messenger RNA at ED-A, ED-B and CS1 regions in human liver tumors. Cancer Res. 53:2005-2011, 1993.

Pardee,A.B. G_1 events and regulation of cell proliferation. Science. 246:603-608, 1989.

Parker,S.L., Tong,T., Bolden,S. And Wingo,P.A. Cancer statistics, 1997. CA. A Cancer J. for Clinicians 47:5-27, 1997.

Patel,R.S., Odermatt,E., Schwarzbauer,J.E. and Hynes,R.O. Organization of the fibronectin gene provides evidence for exon shuffling during evolution. EMBO J. 6:2565-2572, 1987.

Paulus,W., Huettner,C. and Tonn,J.C. Collagens, integrins and the mesenchymal drift in glioblastomas: a comparison of biopsy specimens, spheroid and early monolayer cultures. Int. J. Cancer. 58:841-846, 1994.

De Pauw-Gillet,M.C.A.J., Christiane,Y.M., Foidart,J.M. and Bassleer,R.J.B. Analysis of tridimensional mixed cultures of mouse B16 melanoma cells and 3T3 fibroblasts. Anticancer Res. 8:153-160, 1988.

Peña,L., Nieto,A., Perez Alenza,M.D., Rodriguez,A., Sanchez,M.A. and Castaño,M. Expression of fibronectin and its integrin receptor $\alpha_5\beta_1$ in canine mammary tumours. Res. Veterinary Sci. 57:358-364, 1994.

Petersen,O.W., Ronnov-Jessen,L., Howlett,A.R. and Bissell,M.J. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proc. Natl. Acad. Sci. USA. 89:9064-9068, 1992.

Pignatelli,M., Hanby,A.M. and Stamp,G.W.H. Low expression of β_1, α_2, and α_3 subunits of VLA integrins in malignant mammary tumours. J. Pathol. 165:25-32, 1991.

Plopper,G.E., McNamee,H.P., Dike,L.E., Bojanowski,K. and Ingber,D.E. Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. Mol. Biol. Cell. 6:1349-1365, 1995.

Polyak,K., Kato.J., Solomon,M.J., Sherr,C.J., Massagué,J., Roberts,J.M. and Koff,A. p27^{Kip1}, a cyclin-Cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. Genes Devel. 8:9-22, 1994.

Ponzetto,C., Bardelli,A., Zhen,Z., Maina,F., dalla Zonca,P., Giordano,S., Graziani,A., Panayotou,G. and Comoglio,P.M. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell. 77:261-271, 1994.

Potts,J.R. and Campbell,I.D. Fibronectin structure and assembly. Curr. Opin. Cell Biol. 6:648-655, 1994.

Pullan,S., Wilson,J., Metcalfe,A., Edwards,G.M., Goberdhan,N., Tilly,J., Hickman,J.A., Dive,C. and Streuli,C.H. Requirement of basement membranes for the suppression of programmed cell death in mammary epithelium. J. Cell Sci. 109:631-642, 1996.

Raghow,R. The role of extracellular matrix in postinflammatory wound healing and fibrosis. FASEB J. 8:823-831, 1994.

Rahimi,N, Saulnier,R., Nakamura,T., Park,M. and Elliott,B.E. Role of hepatocyte growth factor in breast cancer: a novel mitogenic factor secreted by adipocytes. DNA Cell Biol. 13:1189-1197, 1994.

Rahimi,N., Tremblay,E., McAdam,L., Park,M., Schwall,R. and Elliott,B.E. Identification of hepatocyte growth factor autocrine loop in a murine mammary carcinoma. Cell Growth and Diff. 7:263-270, 1996a.

Rahimi,N., Tremblay,E. and Elliott,B.E. Phosphatidylinositol 3-kinase activity is required for hepatocyte growth factor-induced mitogenic signals in epithelial cells. J. Biol. Chem. 271:24850-24855, 1996b.

Rak,J., Mitsuhashi,Y., Erdos,V., Huang,S., Filmus,J. and Kerbel,R.S. Massive programmed cell death in intestinal epithelial cells induced by three-dimensional growth conditions: suppression by mutant c-H-ras oncogene expression. J. Cell Biol. 131:1587-1598, 1995.

Rankin,S., Hooshmand-Rad,R., Claesson-Welsh,L. and Rozengurt,E. Requirement for phosphatidylinositol 3'-kinase activity in platelet-derived growth factor-stimulated tyrosine phosphorylation of p125 focal adhesion kinase and paxillin. J. Biol. Chem. 271:7829-7834, 1996.

Re,F., Zanetti,A., Sironi,M., Polentarutti,N., Lanfrancone,L., Dejana,E. and Colotta,F. Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. J. Cell Biol. 127:537-546, 1994.

Restucci,B., De Vico,G. and Maiolino,P. Expression of β_1 integrin in normal, dysplastic and neoplastic canine mammary gland. J. Comp. Path. 113:165-173, 1995.

Reynisdóttir,I., Polyak,K., Iavarone,A. and Massagué,J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-β. Genes Devel. 9:1831-1845, 1995.

Ridley,A.J. and Hall,A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell. 70:389-399, 1992.

Riikonen,T., Koivisto,L., Vihinen,P. and Heino,J. Transforming growth factor-β regulates collagen gel contraction by increasing $\alpha_2\beta_1$ integrin expression in osteogenic cells. J. Biol. Chem. 270:376-382, 1995a.

Riikonen,T., Westermarck,J., Koivisto,L., Broberg,A., Kähäri,V.M. and Heino,J. Integrin $\alpha_2\beta_1$ is a positive regulator of collagenase (MMP-1) and collagen $\alpha1$ (I) gene expression. J. Biol. Chem. 270:13548-13552, 1995b.

Rizzino,A., Ruff,E. and Rizzino,H. Induction and modulation of anchorage-independent growth by platelet-derived growth factor, fibroblast growth factor and transforming growth factor-$\beta1$. Cancer Res. 46:2816-2820, 1986.

Roberts,A.B., McCune,B.K. and Sporn,M.B. TGF-β:regulation of extracellular matrix. Kidney Inter. 41:557-559, 1992.

Rodgers,H.F., Lavranos,T.C., Vella,C.A. and Rodgers,R.J. Basal lamina and other extracellular matrix produced by bovine granulosa cells in anchorage-independent culture. Cell Tissue Res. 282:463-471, 1995.

Rojiani,M.V., Finlay,B.B., Gray,V. and Dedhar,S. *In vitro* interaction of a polypeptide homologous to human Ro/SS-A antigen (calreticulin) with a highly conserved amino acid sequence in the cytoplasmic domain of integrin α subunits. Biochem. 30:9859-9866, 1991.

Roos.,E. Cellular adhesion, invasion and metastasis. Biochim. Biophys. Acta. 738:263-284, 1983.

Rosales,C., O'Brien,V., Kornberg,L. and Juliano,R.L. Signal transduction by cell adhesion receptors. Biochim. Biophys. Acta. 1242:77-98, 1995.

Rosen,E.M., Nigam,S.K. and Goldberg,I.D. Scatter factor and the c-Met receptor: A paradigm for mesenchymal/epithelial interaction. J. Cell Biol. 127:1783-1787, 1994.

Roskelley,C.D. and Bissell,M.J. Dynamic reciprocity revisited: a continuous, bidirectional flow of information between cells and the extracellular matrix regulates mammary epithelial cell function. Biochem. Cell Biol. 73:391-397, 1995.

Roskelley,C.D., Desprez,P.Y. and Bissell,M.J. Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. Proc. Natl. Acad. Sci. USA. 91:12378-12382, 1994.

Ruoslahti,E. Fibronectin and its receptors. Ann. Rev. Biochem. 57:375-413, 1988.

Ruoslahti,E. Fibronectin in cell adhesion and invasion. Cancer Metastasis Rev. 43-51, 1984.

Ruoslahti,E. Integrins. J. Clin. Invest. 87:1-5, 1991.

Ruoslahti,E., and Pierschbacher,M.D. New perspectives in cell adhesion: RGD and integrins. Science. 238: 491-497, 1987.

Ruoslahti,E. Fibronectin and its $\alpha_5\beta_1$ integrin receptor in malignancy. Invasion and Metasatsis. 95:87-97, 1994.

Ruoslahti,E. and Reed,J.C. Anchorage dependence, integrins,and apoptosis. Cell. 77:477-478, 1994.

Saelman,E.U.M., Keely,P.J. and Santoro,S.A. Loss of MDCK cell $\alpha_2\beta_1$ integrin expression results in reduced cyst formation, failure of hepatocyte growth factor/scatter factor-induced branching morphogenesis, and increased apoptosis. J. Cell Sci. 108:3531-3540, 1995.

Saito,T., Kimura,M., Kawasaki,T., Sato,S. and Tomita,Y. Correlation between integrin $\alpha5$ expression and the malignant phenotype of transitional cell carcinoma. Br. J. Cancer. 73:327-331, 1996.

Saito,Y., Mori,S., Yokote,K., Kanzaki,T., Saito,Y. and Morisaki,N. Phosphatidylinositol 3-kinase activity is required for the activation process of focal adhesion kinase by platelet-derived growth factor. Biochem. Biophys. Res. Comm. 224:23-26, 1996.

Santos,O.F.P. and Nigam,S.K. HGF-inducd tubulogenesis and branching of epithelial cells is modulated by extracellular matrix and TGF-β. Devel. Biol. 160:293-302, 1993.

Saulnier,R., Bhardwaj,B., Klassen,J., Leopold,D., Rahimi,N., Tremblay,E., Mosher,D. and Elliott,B. Fibronectin fibrils and growth factors stimulate anchorage-independent growth of a murine mammary carcinoma. Exp. Cell Res. 222:360-369, 1996.

Schaller,M.D. The focal adhesion kinase. J. Endocrin. 150:1-7, 1996.

Schiro,J.A., Chan,B.M.C., Roswit,W.T., Kassner,P.D., Pentland,A.P., Hemler,M.E., Eisen,A.Z. and Kupper,T.S. Integrin $\alpha_2\beta_1$ (VLA-2) mediates reorganization and contraction of collagen matrices by human cells. Cell. 67:403-410, 1991.

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Schlaepfer,D.D., Hanks,S.K., Hunter,T. and Van der Geer,P. Integrin-mediated signal transduction linked to ras pathway by Grb2 binding to focal adhesion kinase. Nature. 372:786-791, 1994.

Schubert,D. Collaborative interactions between growth factors and the extracellular matrix. TIBS. 2:63-66, 1992.

Schulze,A., Zerfass-Thome,K., Berges,J., Middendorp,S., Jansen-Dürr,P. and Henglein,B. Anchorage-dependent transcription of the cyclin A gene. Mol. Cell. Biol. 16:4632-4638, 1996.

Schwarzbauer,J.E. Identification of the fibronectin sequences required for assembly of a fibrillar matrix. J. Cell Biol. 113:1463-1473, 1991.

Schwarzbauer,J.E., Patel,R.S., Fonda,D. and Hynes,R.O. Multiple sites of alternative splicing of the rat fibronectin gene transcript. EMBO J. 6:2573-2580, 1987.

Schwartz,M.A. and Denninghoff,K. αv integrins mediate the rise in intracellular calcium in endothelial cells on fibronectin even though they play a minor role in adhesion. J. Biol. Chem. 269:11133-11137, 1994.

Schwartz,M.A., Lechene,C. and Ingber,D.E. Insoluble fibronectin activates the Na/H+ antiporter by clustering and immobilizing integrin $\alpha_5\beta_1$, independent of cell shape. Proc. Natl. Acad. Sci. USA. 88:7849-7853, 1991.

Schwartz,M.A. Schaller,M.D. and Ginsberg,M.H. Integrins: Emerging paradigms of signal transduction. Ann. Rev. Cell Biol. 11:549-599, 1995.

Seckl,M.J., Seufferlein,T. and Rozengurt,E. Lysophosphatidic acid-depleated serum, hepatocyte growth factor and stem cell growth factor stimulate colony growth of small cell lung cancer cells through a calcium-independent pathway. Cancer Res. 54:6143-6147, 1994.

Seftor,R.E.B., Seftor,E.A., Gehlsen,K.R., Stetler-Stevenson,W.G., Brown,P.D., Ruoslahti,E. and Hendrix,J.C. Role of the $\alpha_v\beta_3$ integrin in human melanoma cell invasion. Proc. Natl. Acad. Sci. USA. 89:1557-1561, 1992.

Sehgal,I., Baley,P.A. and Thompson,T.C. Transforming growth factor β1 stimulates contrasting responses in metastatic *versus* primary mouse prostate cancer-derived cell lines *in vitro*. Cancer Res. 56:3359-3365, 1996.

Seidman,A.P. Chemotherapy for advanced breast cancer: A current persepective. Sem. Oncol. 23:55-59, 1996.

Seslar,S.P., Nakamura,T. and Byers,S.W. Regulation of fibroblast hepatocyte growth factor/scatter factor expression by human breast carcinoma cell lines and peptide growth factors. Cancer Res. 53:1233-1238, 1993.

Shattil,S.J., O'Toole,T., Eigenthaler,M., Thon,V., Williams,M., Babior,B.M. and Ginsberg,M.H. β_3-endonexin, a novel polypeptide that interacts specifically with the cytoplasmic tail of the integrin β_3 subunit. J. Cell Biol. 131:807-816, 1995.

Shaw,L.M., Turner,C.E. and Mercurio,A.M. The $\alpha_{6A}\beta_1$ and $\alpha_{6B}\beta_1$ integrin variants signal differences in the tyrosine phosphorylation of paxillin and other proteins. J. Biol. Chem. 270:23648-23652, 1995.

Shin,S.I., Freedman,V.H., Risser,R. and Pollack,R. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage-independent growth *in vitro*. Proc. Natl. Acad. Sci. USA. 72:4435-4439, 1975.

Song,Z., Varani,J. and Goldstein,I.J. Expression and function of β_1 integrins on adherent and nonadherent Ehrlich ascites tumor cells. Exp. Cell Res. 218:96-104, 1995.

Soriano,J.V., Pepper,M.S., Nakamura,T., Orci,L. and Montesano,R. Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. J. Cell Sci. 108:413-430, 1995.

D'Souza,B., Berdichevsky,F., Kyprianou,N. and Taylor-Papadimitriou,J. Collagen-induced morphogenesis and expression of the α_2-integrin subunit is inhibited in c-erb B2-transfected human mammary epithelial cells. Oncogene. 8:1797-1806, 1993.

Stackpole,C.W., Kalbag,S.S. and Groszek,L. Aquisition of *in vitro* growth autonomy during B16 melanoma malignant progression is associated with autocrine stimulation by transferrin and fibronectin. In Vitro Cell. Dev. Biol. 31:244-251, 1995.

Stephens,L.E., Sutherland,A.E., Klimanskaya,I.V., Andrieux,A., Meneses,J., Pedersen,R.A. and Damsky,C.H. Deletion of β_1 integrins in mice results in inner cell mass failure and peri-implantation lethality. Genes Devel. 9:1883-1895, 1995.

Stracke,M.L., Murata,J., Aznavoorian,S. and Liotta,L.A. The role of the extracellular matrix in tumor cell metastasis. In vivo. 8:49-58, 1994.

Streuli,C.H., Bailey,N. and Bissell,M.J. Control of mammary epithelial differentiation:basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity. J. Cell Biol. 115:1383-1395, 1991.

Streuli,C.H., Schmidhauser,C., Bailey,N., Yurchenco,P., Skubitz,A.P.N., Roskelley,C.D. and Bissell,M.J. Laminin mediates tissue-specific gene expression in mammary epithelia. J. Cell Biol. 129:591-603, 1995.

Sutton,A.B., Canfield,A.E., Schor,S.L., Grant,M.E. and Schor,A.M. The response of endothelial cells to TGF-β1 is dependent upon cell shape, proliferative state and the nature of the substratum. J. Cell Sci. 99:777-787, 1991.

Suzuki,S. and Naitoh,Y. Amino acid sequence of a novel integrin ß$_4$ subunit and primary expression of the mRNA in epithelial cells. EMBO J. 9:757-763, 1990.

Symington,B.E. Fibronectin receptor modulates cyclin-dependent kinase activity. J. Biol. Chem. 267:25744-25747, 1992.

Symington,B.E. Growth signalling through the alpha5 beta1 fibronectin receptor. Biochem. Biophys. Res. Commun. 208:126-134, 1995.

Taipale,J. and Keski-Oja,J. Hepatocyte growth factor releases epithelial and endothelial cells from growth arrest induced by transforming growth factor-β1. J. Biol. Chem. 271:4342-4348, 1996.

Talhouk,R.S., Bissell,M.J. and Werb,Z. Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. J. Cell Biol. 118:1271-1282, 1992.

Tang,D.G. and Honn,K.V. Adhesion molecules and tumor metastasis: an update. Invasion Metastasis. 14:109-122, 1995.

Tavoloni,N., Inoue,H., Sabe,H. and Hanafusa,H. v-src transformation of rat embryo fibroblasts. Inefficient conversion to anchorage-independent growth involves heterogeneity of primary cultures. J. Cell Biol. 126:475-483, 1994.

Tawil,N.J., Gowri,V., Djoneidi,M., Nip,J., Carbonetto,S. and Brodt,P. Integrin $\alpha_3\beta_1$ can promote adhesion and spreading of metastatic breast carcinoma cells on the lymph node stroma. Int. J. Cancer. 66:703-710, 1996.

Teixeira,M.R., Pandis,N., Gerdes,A.M., Dietrich,C.U., Bardi,G., Andersen,J.A., Graversen,H.P., Mitelman,F. And Heim,S. Cytogenetic abnormalities in an in situ ductal carcinoma and five prophylactically removed breasts from members of a family with hereditary breast cancer. Breast Cancer Res. Treat. 38:177-182, 1996.

O'Toole,T.E., Katagiri,Y., Faull,R.J., Peter,K., Tamura,R., Quaranta,V., Loftus,J.C., Shattil,S.J. and Ginsberg,M.H. Integrin cytoplasmic domains mediate inside-out signal transduction. J. Cell. Biol. 124:1047-1059, 1994.

Travers,M.T., Barrett-Lee,P.J., Berger,U., Luqmani,Y.A., Gazet,J.C., Powles,T.J. and Coombes,R.C. Growth factor expression in normal, benign, and malignant breast tissue. Br. Med. J. 296:1621-1624, 1988.

Uehara,Y. and Kitamura,N. Expression of a human hepatocyte growth factor/scatter factor cDNA in MDCK epithelial cells influences cell morphology, motility, and anchorage-independent growth. J. Cell Biol. 117:889-894, 1992.

Varner,J.A., Emerson,D.A., and Juliano,R.L. Integrin $\alpha_5\beta_1$ expression negatively regulates cell growth: reversal by attachment to fibronectin. Mol. Biol. Cell. 6:725-740, 1995.

Varticovski,L., Harrison-Findik,D., Keeler,M.L. and Susa,M. Role of PI 3-kinase in mitogenesis. Biochim. Biophys. Acta. 1226:1-11, 1994.

Vignon,F., Prebois,C., and Rochefort,H. Inhibition of breast cancer growth by suramin. J. Natl. Cancer Inst. 84:38-42, 1992.

von der Mark,K., von der Mark,H. and Goodman,S. Cellular responses to extracellular matrix. Kidney Inter. 41:632-640, 1992.

Waleh,N.S. Gallo,J. Grant,T.D., Murphy,B.J., Kramer,R.H. and Sutherland,R.M. Selective down-regulation of integrin receptors in spheroids of squamous cell carcinoma. Cancer Res. 54:838-843, 1994.

Walker,R.A., Dearing,S.J. and Gallacher,B. Relationship of transforming growth factor β_1 to extracellular matrix and stromal infiltrates in invasive breast carcinoma. Br. J. Cancer. 69:1160-1165, 1994.

Watt,F.M. The extracellular matrix and cell shape. TIBS. 11:482-485, 1986.

Welch,D.R., Fabra,A. and Nakajima,M. Transforming growth factor-β stimulates mammary adenocarcinoma cell invasion and metastatic potential. Proc. Natl. Acad. Sci. USA. 87:7678-7682, 1990.

Weiner,T.M., Liu,E.T., Craven,R.J. and Cance,W.G. Expression of focal adhesion kinase gene and invasive cancer. Lancet. 342:1024-1025, 1993.

Weiss,L., Mayhew,E., Glaves Rapp,D. and Holmes,J.C. Metastatic inefficiency in mice bearing B16 melanomas. Br. J. Cancer. Suppl. 45-53, 1982.

Werb,Z., Tremble,P.M., Behrendtsen,O., Crowley,E. and Damsky,C.H. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. 109:877-889, 1989.

Winklbauer,R. and Stoltz,C. Fibronectin fibril growth in the extracellular matrix of the Xenopus embryo. J. Cell Sci. 108:1575-1586, 1995.

Wong,S.Y., Purdie,A.T. and Han,P. Thrombospondin and other possible related matrix proteins in malignant and benign breast disease. Am. J. Pathol. 140:1473-1482, 1992.

Wu,C., Bauer,J.S., Juliano,R.L. and MacDonald,J.A. The $\alpha_5\beta_1$ integrin fibronectin receptor, but not the $\alpha5$ cytoplasmic domain, functions in an early and essential step in fibronectin matrix assembly. J. Biol. Chem. 268:21883-21888, 1993.

Wu,C., Chung,A.E. and McDonald,J.A. A novel role for $\alpha_3\beta_1$ integrins in extracellular matrix assembly. J. Cell Sci. 108:2511-2523, 1995.

Yamada,K.M. and Miyamoto,S. Integrin transmembrane signaling and cytoskeletal control. Curr. Opin. Cell Biol. 7:681-689, 1995.

Yamaguchi,Y., Mann,D.M. and Ruoslahti,E. Negative regulation of transforming growth factor-β by the proteoglycan decorin. Nature. 346:281-284, 1990.

Yonezawa,I., Kato,K., Yagita,H., Yamauchi,Y. and Okumura,K. VLA-5-mediated interaction with fibronectin induces cytokine production by human chondrocytes. Biochem. Biophys. Res. Comm. 219:261-265, 1996.

Yao,R. and Cooper,G.M. Requirement for phosphatidylinositol 3-kinase in the prevention of apoptosis by nerve growth factor. Science 267:2003-2006, 1995.

Yao,R. and Cooper,G.M. Growth factor-dependent survival of rodent fibroblasts requires phosphatidylinositol 3-kinase but is independent of p70^{S6K} activity. Oncogene. 13:343-351, 1996.

Ye,J., Xu,R.H., Taylor-Papadimitriou,J. and Pitha,P.M. Sp1 binding plays a critical role in erb-B2- and v-ras-mediated down-regulation of α_2-integrin expression in human mammary epithelial cells. Mol. Cell. Biol. 16:6178-6189, 1996.

Zhang,K., Vuori,K., Reed,J.C. and Ruoslahti,E. The $\alpha_5\beta_1$ integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. Proc. Natl. Acad. Sci. USA. 92:6161-6165,1995.

Zhang,Z., Vuori,K., Wang,H.G., Reed,J.C. and Ruoslahti,E. Integrin activation by R-ras. Cell. 85:61-69, 1996.

Zheng,M., Fang,H., Tsuruoka,T., Tsuji,T., Sasaki,T. and Hakomori,S. Regulatory role of G_{M3} ganglioside in $\alpha_5\beta_1$ integrin receptor for fibronectin-mediated adhesion of FUA169 cells. J. Biol. Chem. 268:2217-2222, 1993.

Zhu,X. and Assoian,R.K. Integrin-dependent activation of MAP kinase: a link to shape-dependent cell proliferation. Mol. Biol. Cell. 6:273-282, 1995.

Zhu,X., Ohtsubo,M., Böhmer,R.M., Roberts,J.M. and Assoian,R.K. Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein. J. Cell Biol. 133:391-403, 1996.

Zutter,M.M., Mazoujian,G. and Santoro,S.A. Decreased expression of integrin adhesive protein receptors in adenocarcinoma of the breast. Am. J. Pathol., 137:863-870, 1990.

Zutter,M.M. and Santoro,S.A. Widespread histologic distribution of the $\alpha_2\beta_1$ integrin cell-surface collagen receptor. Am. J. Pathol. 137:113-120, 1990.

Zutter,M.M., Santoro,S.A., Staatz,W.D. and Tsung,Y.L. Re-expression of the $\alpha_2\beta_1$ integrin abrogates the malignant phenotype of breast carcinoma cells. Proc. Natl. Acad. Sci. USA. 92:7411-7415, 1995.

Zwijsen,R.M.L., Klompmaker,R., Wientjens,E.B.H.G.M., Kristel,P.M.P., van der Burg,B. and Michalides,R.J.A.M. Cyclin D1 triggers autonomous growth of breast cancer cells by governing cell cycle exit. Mol. Cell. Biol. 16:2554-2560, 1996.

K-*ras* modulates the cell cycle via both positive and negative regulatory pathways

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The effect of activated human K-*ras* on cell cycle proteins was studied by use of a stable MCF-7 transfectant expressing inducible activated K-*ras* under the control of a tetracycline (Tet)-responsive promoter. Induction of activated K-*ras* by Tet withdrawal accelerated cell growth and entry into S-phase. To understand the mechanism(s) by which activated K-*ras* exerts its effect on the cell cycle, expression of both cell cycle stimulatory proteins as well as cell cycle inhibitors was examined. Upon induction of activated K-*ras*, several cell cycle stimulators were up-regulated, including cyclins A, D3, and E, and the E2F family of transcription factors, which was accompanied by increased cyclin A-associated kinase activity and E2F transcriptional activity, respectively. Up-regulation of cyclin A occurred at the transcriptional level and in a serum-dependent manner. Furthermore, induction of activated K-ras down-regulated p27^{Kip1} and up-regulated p53. Up-regulation of p53 was correlated with enhanced p53 transactivation and accompanied by up-regulation of p21^{Waf1} and Gadd 45, two p53 effectors and negative cell cycle regulators. In addition, activated K-*ras* up-regulates *bcl-2* but has no effect on *bax* or *bcl-x* expression. Taken together, these data indicate that activated K-ras affects the cell cycle by modulating both positive and negative cell cycle regulatory pathways.

Keywords: ras; cell cycle; gene regulation

Introduction

The cell cycle of mammalian cells is regulated by a very complex machinery, of which cyclins and cyclin dependent protein kinases (CDKs) play essential roles (reviewed in Pines, 1995; Nigg, 1995). Multiple cyclin-CDK complexes undergo changes in the kinase and cyclin moieties which are believed to drive the cell cycle from one stage to another. An important element of this cyclin regulated cell cycle is the rapid induction of cell cycle phase specific cyclin expression and facile destruction of these cyclins as cells progress through the next phase of the cell cycle. In mammalian cells, the D-type cyclins (reviewed in Sherr, 1995), in association with the cyclin-dependent kinases cdk4 or cdk6, regulate the progression of the G$_1$ phase of the cell cycle mainly by participating in the phosphorylation of the retinoblastoma gene product pRb (reviewed in Weinberg, 1995). As a result of phosphorylation of pRb, free E2F, of which five family members have

been identified to date (Sardet *et al.*, 1995; Hijmans *et al.*, 1995), is released from complexes containing E2F and the hypophosphorylated form of pRb. The 'free' E2F, which is present as a heterodimer with its binding partner DP-1 or DP-2 (Wu *et al.*, 1995), is the active transcription factor that promotes the transcription of E2F target genes such as dihydrofolate reductase (DHFR) (Blake and Azizkhan, 1989), thymidylate synthase (TS) (Johnson, 1994) and other genes necessary for entry into and progression through the S phase of the cell cycle (Adams and Kaelin, 1995; DeGregori *et al.*, 1995). Cyclin A and the B-type cyclins (cyclins B1 and B2), in complexes with CDK2 and CDC2, respectively, are essential for S-phase progression and for G$_2$/M phase transition (reviewed in Pines and Hunter, 1992).

The mammalian cell cycle is negatively regulated mostly through action of cyclin dependent kinase inhibitors (CDKIs) (reviewed in Sherr and Roberts, 1995), many of which have been identified recently. The most important CDKIs include p21^{Waf1}, a universal CDK inhibitor (Xiong *et al.*, 1993) whose expression is regulated by the p53 tumor suppressor protein (el-Deiry *et al.*, 1993); p16^{INK4}, a specific inhibitor of cyclin D/CDK4/6 that is identified recently as a tumor suppressor gene product (Marx, 1994); and p27^{Kip1}, which is involved in cellular response to cAMP and other antiproliferative signals (Polyak *et al.*, 1994; Kato *et al.*, 1994). p21^{Waf1} plays a pivotal role in p53-mediated cell cycle arrest upon stress such as DNA damage (Brugarolas *et al.*, 1995; Macleod *et al.*, 1995).

The *ras* oncogenes (including H-, K- and N-*ras*), which are activated by point mutations, are implicated in carcinogenesis (reviewed in Barbacid, 1990; Rodenhuis, 1992). About 30% of all human tumors are associated with *ras* mutations and up to 95% of human pancreatic cancers contain K-*ras* mutations (Mangues and Pellicer, 1992; Rodenhuis, 1992). Ras proteins play central roles in receptor-mediated signal transduction pathways that control cell proliferation and differentiation (Khosravi-Far and Der, 1994; Medema and Bos, 1993). Increasing evidence supports the notion that one of the ultimate targets of the *ras* signaling pathway is the nuclear transcription machinery including the transcription factors c-Jun and Ets, whose activity has been shown to be regulated by oncogenic *ras* (Westwick *et al.*, 1994; Yang *et al.*, 1996). Therefore, *ras* plays an essential role in transducing external signals (such as growth factors or other mitogens) to the nucleus.

Earlier work demonstrated that microinjection of Ras protein into quiescent cells induces DNA synthesis (Stacey and Kung, 1984), suggesting that the *ras* signaling pathway is directly linked to the G$_1$/S transition of the cell cycle. The link

between *ras* and the cell cycle has been strengthened by more recent reports showing that activated *ras* induces cyclin D_1 in NIH3T3 cells (Winston *et al.*, 1996; Filmus *et al.*, 1994) and that the *raf* kinase, a putative downstream target of *ras*, interacts with and regulates the activity of cdc25 (Galaktionov *et al.*, 1995), a phosphatase involved in regulation of cdc2, an essential G_2/M kinase. However, the direct roles of *ras* in regulation of the very complex cell cycle machinery is yet to be elucidated. The current study was undertaken to study the effect of activated *ras* on the cell cycle and on the expression of cell cycle related genes and to understand the functional roles of *ras* in relationship to cell cycle control. Ultimately, the information obtained in this study should help to identify the molecular target(s) of activated *ras* and provide guidance for chemotherapy of tumors containing activated *ras*.

Results

Inducible expression of an activated K-ras in MCF-7 cells

In order to minimize the likelihood of artifacts caused by clonal variation (which is reduced, if not eliminated, by use of an inducible expression system), a common problem associated with stable transfections using non-inducible expression vectors, MCF-7 cells were cloned and cells from a *single* clone (#2) (which harbors normal *ras* genes as determined by sequencing analysis (data not shown)) were used to obtain stable transfectants expressing inducible human activated K-*ras* under the control of a tetracycline-responsive promoter. After selection with G418, one clone designated as M2TK4 was obtained which contained the activated K-*ras* gene, as shown by PCR using a primer specific for the CMV promoter and another specific for exon 1 of the K-*ras* gene (data not shown). This PCR step was necessary because the plasmid in which the K-*ras* gene resides does not have a selectable marker. Western blotting analysis using a K-*ras* specific antibody revealed that expression of the activated K-*ras* was induced by Tet withdrawal (lane 1, Figure 1) as compared to the level of expression in the presence of Tet (lane 2). The latter most likely represented the endogenous level of K-*ras* expression rather than the 'leakiness' of the system as the level of K-*ras* expression in M2TK4 cells in the presence of Tet was comparable to that in non-transfected MCF-7 cells (data not shown). These data indicate that K-*ras* expression can be effectively regulated (turned on or off) by Tet.

Effect of activated K-ras expression on cell growth and the cell cycle distribution

Although induction of K-*ras* expression in M2TK4 cells had no effect on cell morphology (data not shown), induction of activated K-*ras* did lead to accelerated cell growth as shown in Figure 2a. The doubling times of M2TK4 cells grown in media containing 10% Fetal Bovine Serum (FBS) (Figure 2a, left panel) and in the presence or absence of Tet were 29 and 24 h, respectively. When these cells

were grown in a low serum (0.1%) medium, there was a clear acceleration of cell growth when activated K-*ras* was induced by Tet withdrawal (Figure 2a, right panel), although the overall cell growth was much slower under these conditions compared to normal growth conditions (i.e. 10% serum). The effect of activated K-*ras* on cell cycle (i.e. % of cells in S phase) was measured by use of pulse-chase labeling with BrdU followed by flow cytometric analysis. As shown in Figure 2b, induction of activated K-*ras* in M2TK4 cells led to an increased percentage of cells in S phase (from 26% to 35%). These data indicated that activated K-*ras* can accelerate both cell growth and cell cycle (i.e. G_1-S transition). Careful examination of Figure 2b indicated that there appeared to be a sub-fraction of cells (indicated by the arrow) which were in G_1 phase but BrdU-positive. This sub-population of cells was not evident in M2TK4 cells growing in the presence of Tet. One possible explanation for this observation is that the BrdU-positive G_1 cells were those that were in very late S-phase and were about to enter G_2/M, that had progressed through G_2/M and then returned to G_1 phase of the cell cycle. These data raise the possibility that in addition to acceleration of G_1/S transition, activated K-*ras* may play a role in acceleration of G_2/M transition as well.

Effect of activated K-ras on expression of certain S-phase enzymes

Since induction of activated K-*ras* in M2TK4 cells stimulated cell growth and accelerated the cell cycle (Figure 2), Western blotting analysis was performed to determine whether the increased growth rate and accelerated cell cycle are accompanied by increased levels of enzymes such as dihydrofolate reductase and thymidylate synthase which are essential for DNA replication and cell growth. When activated K-*ras* was induced by tetracycline withdrawal in cells grown in 10% FBS, there was a slight increase in the level of

A

B

Control   M2TK4

Tet	−	+
%S	35	26
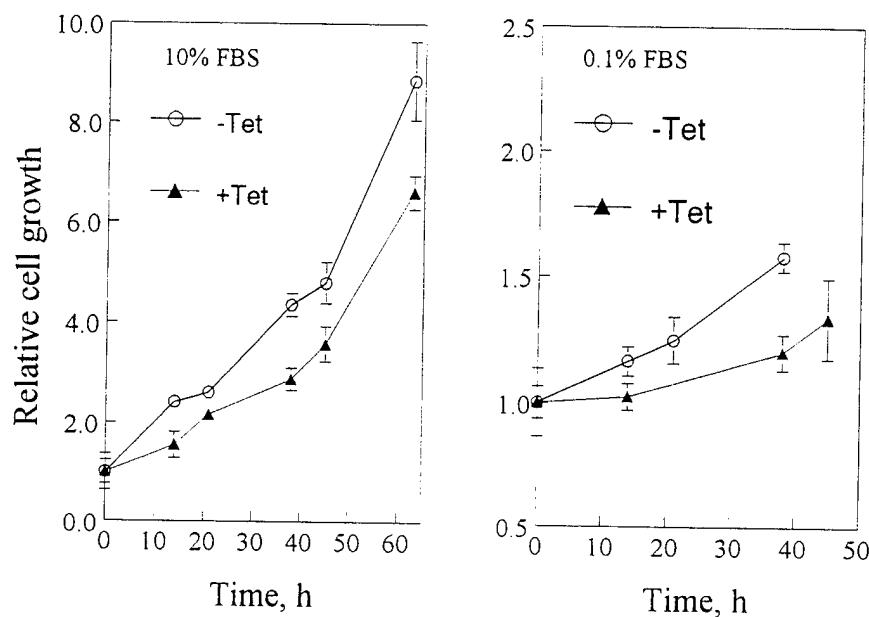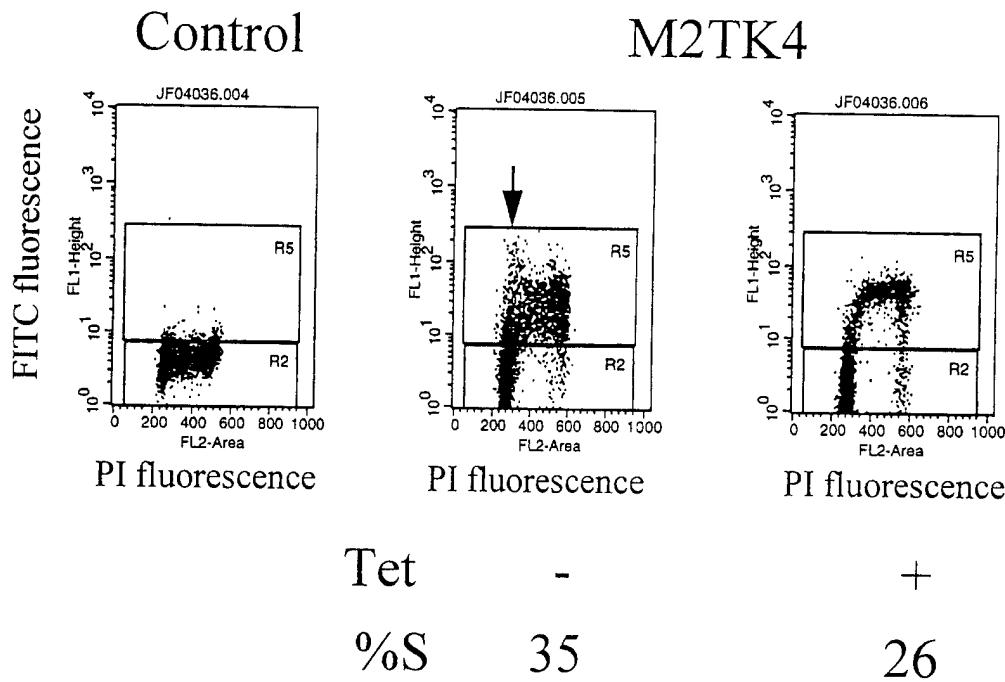
Figure 2 Effect of activated K-*ras* on cell growth and the cell cycle. (a) Cell growth measurement. M2TK4 cells (mid-log phase) that have been maintained in RPMI 1640 medium containing or lacking Tet (1 µg ml) were plated in a series of 96-well plates, which contain media supplemented with either 10% (at 2000 cells per well) or 0.1% FBS (at 5000 cells per well), with or without Tet added, respectively. The cells were incubated at 37°C for 24 h to allow cell attachment. From this point (as time 0 h), and at the indicated time intervals, cell growth was measured by a standard sulforodamine B binding assay as described previously (Fan *et al.*, 1997). The results shown are ratios of cell growth at various time points to that at time 0 h and are means ± standard deviations from eight experiments. (b) Cell cycle analysis. M2TK4 cells, maintained in media containing Tet (1 µg ml), were plated in media (supplemented with 10% serum) containing or lacking Tet (1 µg ml). After growth for 72 h, the cells were labeled with 10 µM Bromodeoxyuridine (BrdU) for 3 h and then collected by trypsinization. After fixation with 70% ethanol, followed by denaturation with 2N HCl, labeling with anti-BrdU-FITC conjugates, and staining with 5 µg ml propidium iodide, the cells were subjected to flow cytometric analysis (see the Materials and methods section for further details). Relative FITC fluorescence (vertical axis) was plotted as a function of DNA contents (PI fluorescence, horizontal axis). Percent of cells in S phase was calculated. The background FITC fluorescence was accessed from a control experiment (left, b) in which wild type MCF-7 cells were processed in the same manner except that the cells were not labeled with BrdU. The results represent one of two independent experiments

TS protein (compare lanes 1 and 2, Figure 3b): the level of DHFR expression was not changed (lanes 1 and 2, Figure 3a). When M2TK4 cells growing in the presence or absence of Tet were exposed to a low serum (0.1% FBS) medium for 24 h, however, there was a significant increase in the levels of both DHFR and TS expression upon Tet withdrawal (compare lanes 3 and 4 in Figure 3a and b). It was reported previously (Graham *et al.*, 1986) that MCF-7 cells may contain high levels of endogenous (normal) ras, which can be activated by serum factors presumably through growth factor receptor pathways. Therefore, when cells are grown in media containing a high concentration (10%) of serum, the effect of the activated K-*ras* transgene (i.e. the transfected activated K-*ras*) may not be apparent unless the cells were subjected to treatment with media containing a low concentration (0.1%) of serum, a condition in which the effect of endogenous ras is greatly reduced if not eliminated. For these reasons, in the subsequent studies (see below), the effect of activated K-*ras* will be studied both in cells grown in regular media (containing 10% serum) and in cells that have been subjected to an additional serum deprivation treatment (with media containing 0.1% serum).

Differential regulation of the D-type cyclins by the activated K-ras

Since D-type cyclins, which are composed of three members (D1, D2, D3), are known to play essential roles in G_1 to S transition in the cell cycle (Sherr, 1995), it was of interest to determine whether activated K-*ras* mediates this cell cycle acceleration through regulation of any or all of these D-type cyclins. This was accomplished by Western blotting analysis, using antibodies specific for each member of the D-cyclin family, of protein extracts from M2TK4 cells grown in the presence or absence of Tet. As shown in Figure 4, when activated K-*ras* was induced by Tet withdrawal and cells were grown in medium containing 10% serum, there was a slight increase in the level of cyclin D3 expression compared to cells grown in the presence of Tet (lanes 1 and 2, Figure 4a). However, the level of cyclin D3 expression was significantly upregulated upon induction of the activated K-*ras* by Tet withdrawal when cells were subjected to a 24 h serum deprivation with medium containing 0.1% serum before being collected for protein extraction. The up-regulation of cyclin D3 by activated K-*ras* correlated with cyclin D3-associated kinase activity in an experiment in which an anti-cyclin D3 antibody was used to immunoprecipitate the kinase(s) complexed with cyclin D3 and Histone H1 was used as the substrate (data not shown). The effect of activated K-*ras* on the levels of cyclin D1 and cyclin D2 expression was analysed in a similar manner. As shown in Figure 4b and c, no obvious difference in the level of either cyclin D1 or cyclin D2 expression was observed when cells were grown either in the presence or in the absence of Tet, with or without the serum deprivation step described above. These data show that induction of activated K-*ras* upregulated only one member of the D-type cyclin family – cyclin D3 – while having no effect on cyclins D1 and D2.
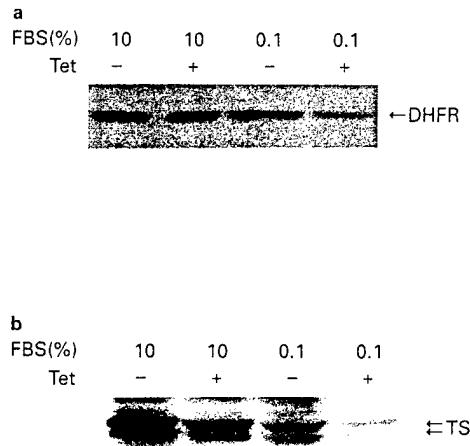
Figure 3 Effect of activated K-*ras* on expression of two S-phase enzymes. M2TK4 cells (mid-log), maintained in the presence of Tet, were grown to mid-log phase (a 48 h incubation) in media containing or lacking Tet (2 μg/ml), using multiple tissue culture flasks. Cells from one half of these flasks (growing in the presence or absence of Tet) were subjected to an additional 24 h serum deprivation by incubating cells with media containing 0.1% FBS, with or without Tet added, respectively. Cells were collected by trypsinization and proteins from the total cellular extract were subjected to SDS–PAGE followed by Western blotting with a polyclonal anti-DHFR antibody (a) or anti-TS antibody (b). Positions of DHFR and TS are indicated by arrows. The figure represents one of three to five independent experiments
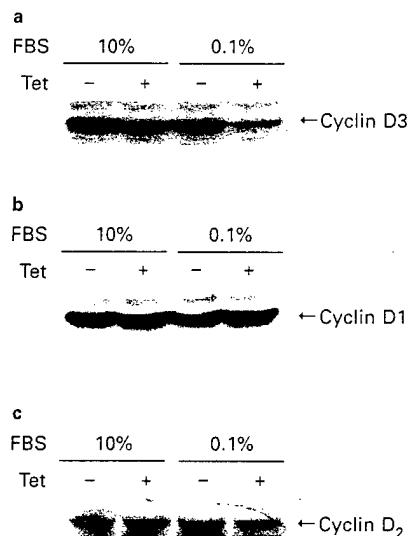
Figure 4 Differential regulation of the D-type cyclins by activated K-*ras*. M2TK4 cells were grown to mid-log phase, in the presence or absence of Tet, with or without a serum deprivation step, as described in the legend for Figure 3. Cells were collected by trypsinization and proteins from the total cellular extract were subjected to SDS–PAGE followed by Western blotting with a polyclonal anti-cyclin D3 antibody (a), a monoclonal anti-cyclin D1 antibody (b) or a polyclonal anti-cyclin D2 antibody (c). Positions of cyclins D1, D2 and D3 are indicated by arrows. The figure represents one of three different experiments

Induction of activated K-ras leads to upregulation of the E2F family of transcription factors

Since the E2F family of transcription factors play a pivotal role in progression through the G_1 phase of the cell cycle and entry into S phase (Johnson *et al.*, 1993), it was of interest to determine whether activated K-*ras* mediates cell cycle acceleration through regulation of any or all of the E2F family of transcription factors, of which five members have been identified to date. Of these five members, E2F-1, 2, and 3 preferentially bind to pRb while E2F-4 and E2F-5 preferentially bind the pRb-related proteins p107 and p130, respectively (Beijersbergen *et al.*, 1994; Hijmans *et al.*, 1995). Western blotting analysis, using an antibody specific for E2F-1 showed that induction of activated K-*ras* by Tet withdrawal resulted in a significant increase in the level of E2F-1 expression (Figure 5a, compare lanes 1 and 2). This induction of E2F-1 by the activated K-*ras* appeared to be more profound when cells growing in the presence or absence of Tet were subjected to a 24 h serum deprivation (as described above) (Figure 5a, compare lanes 3 and 4). Although serum starvation led to a decrease in overall E2F-1 expression in cells exposed or not exposed to Tet, significant expression of E2F-1 was evident when activated K-*ras* was induced in serum-starved cells while E2F-1 expression was almost non-detectable in these cells under non-induced conditions. Effect of induction of activated K-*ras* on expression of E2F-4 and E2F-5, other two distinct

members of the E2F family of transcription factors, was studied in the same manner using antibodies specific for E2F-4 and E2F-5, respectively. As shown in Figure 5b and c, induction of activated K-*ras* by Tet withdrawal resulted in a significant increase in the levels of E2F-4 and E2F-5 either with or without serum deprivation. Interestingly, serum deprivation did not appear to have much effect on the overall expression of these E2F species as compared to E2F-1. These data clearly indicate that induction of activated K-*ras* leads to upregulation of all major forms of the E2F transcription factors.

Induction of the E2F transcription factors by activated K-ras is consistent with increased E2F transcriptional activity

The transcriptional activity of members of the E2F family of transcription factors is regulated by multiple cell cycle-related events, such as increased expression (transcriptionally or post-transcriptionally) (Slansky *et al.*, 1993), phosphorylation (for E2F-1, 2 and 3) by cyclin A/cdk2 complexes (Xu *et al.*, 1994) and complex formation with pRb or pRb-related proteins p107 and p130. Therefore, increased expression of the E2F protein may not necessarily lead to increased transcriptional activity. The E2F transcriptional activity was measured by transient transfection of M2TK4 cells with a plasmid containing the CAT reporter gene driven by a E2F-responsive promoter. As shown in Figure 6,
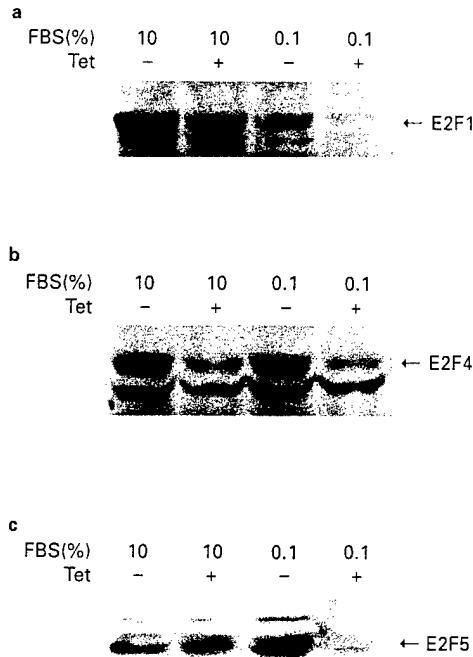
Figure 5 Effect of activated K-*ras* on expression of members of the E2F family of transcription factors. M2TK4 cells were grown to mid-log phase, in the presence or absence of Tet, with or without a serum deprivation step, as described in the legend for Figure 3. Cells were collected by trypsinization and proteins from the total cellular extract were subjected to SDS–PAGE followed by Western blotting with specific monoclonal antibody against E2F-1 (a), E2F-4 (b) or E2F-5 (c). Positions of E2F-1, -2 and -5 are indicated by arrows. The figure represents one of at least three different experiments
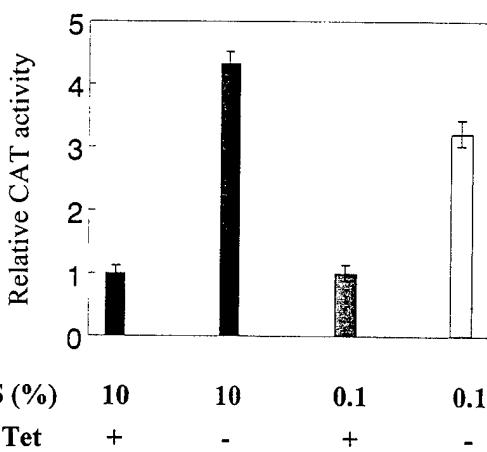
Figure 6 Effect of activated K-*ras* on E2F transcriptional activity. M2TK4 cells were grown to ca. 40–50% confluency, in the presence or absence of Tet, in multiple tissue culture dishes. After 24 h growth, cells were co-transfected, using lipofection, with pE2FCAT (which contains an E2F responsive minimal TK promoter driving the expression of the CAT reporter gene) and pSV2luc (which contains the luciferase gene driven by the SV40 promoter). After an additional 72 h growth, cells from one half of these dishes (growing in the presence or absence of Tet) were subjected to 24 h serum deprivation by incubating cells with media containing 0.1% FBS, with or without Tet added, respectively. Cells were collected and assayed for both CAT and luciferase activities. The results represent the means±s.e. of relative CAT activities (after correction for transfection efficiency using luciferase activities) from three experiments. For details, see the Materials and methods section

when activated K-*ras* was induced by Tet withdrawal. there was a fourfold increase in overall E2F transcriptional activity (as indicated by relative CAT activity after correction for transfection efficiency using a separate reporter plasmid containing the luciferase gene) as compared to the result obtained from cells grown in the presence of Tet. Similar results were obtained when transfected cells growing in the presence or absence of Tet were subjected to serum deprivation (0.1% serum, 24 h) before being collected for the CAT assay. These results indicated that induction of the activated K-*ras* leads to increased E2F transcriptional activity.

Up-regulation of cyclins A and E by activated K-ras

Although cyclin A, initially characterized as a 'mitotic cyclin' (Walker and Maller, 1991), is considered to function as an S-phase cyclin (Zindy et al., 1992; Pagano et al., 1992), recent work (Carbonaro-Hall et al., 1993; Rosenberg et al., 1995) suggests that cyclin A activity may also be essential for G_1/S progression. Therefore, the effect of activated K-*ras* on expression of both cyclin A and cyclin E (a G_1 cyclin; Ohtsubo et al., 1995), was studied. Western blotting analysis using

a
FBS(%)   10   10   0.1   0.1
Tet   –   +   –   +

← Cyclin A

b
Tet   –   +

⇆ Histone H1

c
FBS(%)   10   10   0.1   0.1
Tet   –   +   –   +

← Cyclin E

Figure 7 Regulation of cyclins A and E by activated K-*ras*. (**a** and **c**) M2TK4 cells were grown to mid-log phase, in the presence or absence of Tet, with or without a serum deprivation step, as described in the legend for Figure 3. Cells were collected by trypsinization and proteins from the total cellular extract were subjected to SDS–PAGE followed by Western blotting with specific monoclonal antibody against cyclin A (a) or cyclin E (c). Positions of cyclins A and E are indicated by arrows. The figure represents one of three similar experiments. (**b**) Histone H1 kinase assay. M2TK4 cells (mid-log), maintained in the presence of Tet, were grown to mid-log phase (a 48 h incubation) in media containing or lacking Tet (2 μg/ml). Cells were collected by trypsinization and the total cellular extract (containing 200 μg protein) were subjected to immunoprecipitation with anti-cyclin A antibody-agarose conjugates followed by Histone H1 assay as described in the Materials and methods section. The position of the phosphorylated Histone H1 was indicated by the arrow. This represents one of two similar experiments

a specific antibody against cyclin A (Figure 7a) indicated that induction of activated K-*ras* by Tet withdrawal in M2TK4 cells led to a significant increase in cyclin A protein when cells were grown in media containing 10% serum (with or without Tet) (lanes 1 and 2). However, when cells growing in the presence or absence of Tet were subjected to a 24 h serum deprivation, cyclin A expression became undetectable and induction of K-*ras* failed to upregulate cyclin A.

Cyclin A, like other cyclins, forms active complexes with its corresponding CDKs (i.e. CKD2) to drive cell cycle progression (Devoto et al., 1992). To determine whether upregulation of cyclin A by activated K-*ras* plays a role in cell cycle regulation, cyclin A-associated kinase activity was determined by measuring the ability of the cyclin A immunoprecipitate from total cellular extract to phosphorylate Histone H1 protein. As shown in Figure 7b, when activated K-*ras* was induced by Tet withdrawal and cells were grown in medium containing 10% serum, there was a significant increase in cyclin A-associated kinase activity compared to cells grown in the presence of Tet (lanes 1 and 2).

The effect of activated K-*ras* on expression of cyclin E. a G_1 cyclin, was studied in a similar manner. As shown in Figure 7c. activated K-*ras* also induces cyclin E in cells grown with regular medium (lanes 1 and 2) but not in cells which were serum starved (lanes 3 and 4). These data suggest that activated K-*ras* regulates both cyclin A and cyclin E, and most likely by a different mechanism from that for cyclin D3 in that up-regulation of cyclin A and cyclin E by activated K-*ras* may require the presence of certain serum factors while up-regulation of cyclin D3 does not.

Regulation of cyclin A by activated K-ras occurs at the transcriptional level

Transient transfection of M2TK4 cells with a plasmid containing the cyclin A promoter which drives the expression of the reporter gene luciferase was performed to measure the effect of induction of activated K-*ras* on the promter activity of cyclin A. As shown in Figure 8, induction of activated K-*ras* by Tet withdrawal leads to a significant increase in the promoter activity of cyclin A (as indicated by relative luciferase activity after correction for transfection efficiency using a separate reporter plasmid containing the CAT gene). Similar results were obtained when transfected cells growing in the presence or absence of Tet were subjected to serum deprivation (0.1% serum, 24 h) before being collected for the luciferase assay. These results indicate that the activated K-*ras* regulates cyclin A expression at the transcriptional level.

Induction of activated K-ras leads to down-regulation of p27Kip1

While the E2F family of transcription factors are known to be positive regulators of the cell cycle. inhibitors of cyclins and cyclin dependent kinases such as p27Kip1 are negative regulators of the cell cycle. The earlier observations that overexpression of activated ras. in some special cases, resulted in cell cycle arrest (Pan et al., 1994; Hirakawa and Ruley, 1988) prompted us to examine whether activated K-*ras*, shown to regulate several positive regulators of the cell cycle, can

regulate some of the 'negative' cell cycle regulators. Therefore, the effect of activated K-*ras* expression on p27[Kip1] was examined by Western blotting using specific antibodies against this protein. As shown in Figure 9, induction of activated K-*ras* by Tet withdrawal resulted in a significant decrease in the level of p27[Kip1] expression (compare lanes 1 and 2). This effect (i.e. repression of p27[Kip1] expression by activated K-*ras*) was retained in cells growing in the presence or absence of Tet followed by a 24 h serum deprivation (compare lanes 3 and 4). These data suggest that induction of activated K-*ras* decreases the expression of p27[Kip1].

which could contribute, at least in part, to an accelerated cell cycle.

Induction of activated K-ras leads to upregulation of p53 and its effectors p21[waf1] and Gadd 45

A previous study by Hicks *et al.* (1991) showed that overexpression of an activated *ras* gene in the rat embryo fibroblast cell line REF52 results in growth arrest which may be released by expression of dominant negative mutants of p53, indicating that wild type p53 activity may be essential for ras-mediated growth arrest. Therefore, it was of interest to test whether activated K-*ras* directly regulates the expression of the tumor suppressor protein p53. Western blotting analysis using a specific antibody against p53 showed that induction of activated K-*ras* by Tet withdrawal resulted in a significant increase in the level of p53 expression (compare lanes 1 and 2, Figure 10a). This induction of p53 by the activated K-*ras* appeared to be more profound when cells growing in the presence or absence of Tet were subjected to a 24 h serum deprivation (compare lanes 3 and 4). In order to test whether the up-regulated p53 is functionally linked to the ras-mediated negative regulation of the cell cycle, we examined the effect of activated K-*ras* on two p53 effector proteins, the universal CDK inhibitor p21[waf1], which plays an important role in p53-mediated cell cycle arrest in response to DNA damage (Macleod *et al.*, 1995), and Gadd 45, which has also been shown to inhibit entry of cells into S phase (Carrier *et al.*, 1994) in addition to stimulation of DNA excision repair (Smith *et al.*, 1994). Western blotting analysis showed that induction of activated K-*ras* led to a

FBS (%)	10	10	0.1	0.1
Tet	+	-	+	-
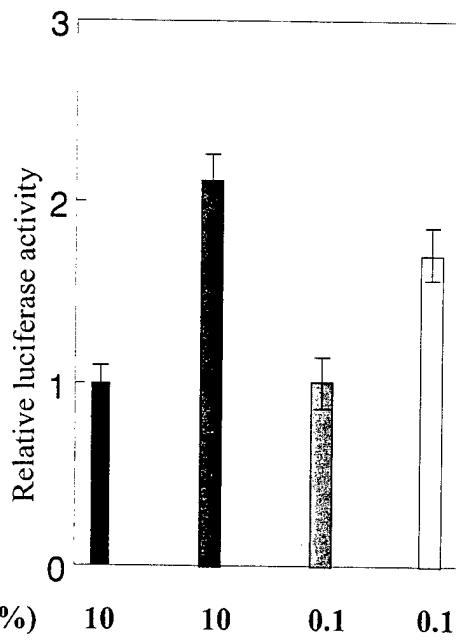
Figure 8 Effect of activated K-*ras* on the transcriptional activity of the cyclin A promoter. The effect of activated K-*ras* on the cyclin A promoter activity was measured as described in the legend for Figure 6 except that, instead of using pE2FCAT and pSVluc in the co-transfection experiments, cells were co-transfected with pAluc (which contains the cyclin A promoter driving the expression of the luciferase reporter gene) and pSV2CAT (which contains the CAT gene driven by an SV40 promoter), the results represent the means±s.e. of relative luciferase activities (after correction for transfection efficiency using CAT activities) from three experiments

| FBS(%)	10	10	0.1	0.1
Tet	–	+	–	+

← p27

1   2   3   4
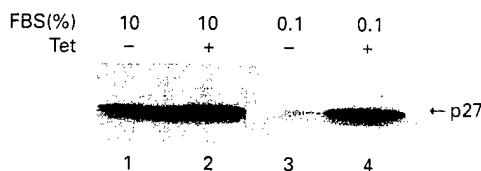
Figure 9 Effect of activated K-*ras* on p27 expression. M2TK4 cells were grown to mid-log phase, in the presence or absence of Tet, with or without a serum deprivation step, as described in the legend for Figure 3. Cells were collected by trypsinization and proteins from the total cellular extract were subjected to SDS – PAGE followed by Western blotting with a polyclonal anti-p27[Kip1] antibody. The position of p27 is indicated by the arrow. The figure represents one of three similar experiments

a

FBS(%)	10	10	0.1	0.1
Tet	–	+	–	+

← p53

b

FBS(%)	10	10	0.1	0.1
Tet	–	+	–	+

← p21[waf1]

c

Tet	–	+
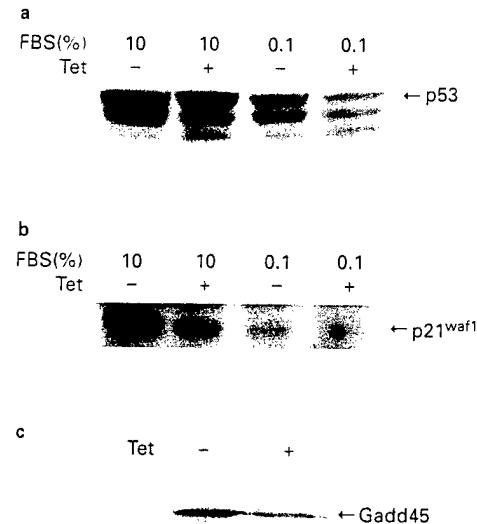
← Gadd45

Figure 10 Effect of activated K-*ras* on expression of p53 and its effectors p21[waf1] and gadd 45. M2TK4 cells were grown to mid-log phase, in the presence or absence of Tet, with or without a serum deprivation step, as described in the legend for Figure 3. Cells were collected by trypsinization and proteins from the total cellular extract were subjected to SDS – PAGE followed by Western blotting with a monoclonal antibody against p53 (a), or polyclonal antibodies against p21[waf1] (b) or Gadd 45 (c). Positions of these proteins are indicated by arrows. The figure represents one of two to four independent experiments

significant increase in the level of p21 expression (compare lanes 1 and 2, Figure 10b). However, when cells (growing in the presence or absence of Tet) were subjected to a 24 h serum deprivation, p21 expression was dramatically reduced and induction of K-*ras* failed to upregulate p21 (lanes 3 and 4, Figure 10b) despite that, as shown above (lanes 3 and 4 of Figure 10a), p53 was induced under these conditions. As shown in Figure 10c, induced expression of activated K-*ras* led to a significant increase in Gadd 45 (compare lanes 1 and 2). These data suggest that (1) Induction of activated K-*ras* increases expression of several negative regulators of the cell cycle such as p53 and its effectors p21^{waf1} and Gadd 45; (2) regulation of p21 by activated K-*ras* may require the presence of certain serum factors, which is similar to the regulation of cyclins A and E by K-*ras*; and (3) Regulation of p21^{waf1} appears to be partially p53-dependent and partially independent.

Upregulation of p53 by activated K-ras correlates with increased p53-dependent transactivation

Previous studies provided evidence that activity of p53 as a transcription factor is regulated by additional mechanisms besides transcriptional regulation of the p53 gene. These additional mechanisms include stability of the protein (Bae *et al.*, 1995), sequestration in the cytoplasm (Moll *et al.*, 1995), tetramer formation (Friedman *et al.*, 1993) and phosphorylation (Meek and Eckhart, 1988). Therefore, it was necessary to determine if the induced p53 protein correlated with p53-mediated transactivation. This was accomplished by transient transfection of M2TK4 cells with a plasmid containing a p53-responsive promoter which controls the expression of the CAT gene (Yamato *et al.*, 1995). As shown in Figure 11, when the activated K-*ras* was induced by Tet withdrawal, there was a fourfold increase in overall p53 transactivation activity (as indicated by relative CAT activity after correction for transfection efficiency using a separate reporter plasmid containing the luciferase gene) as compared to the result obtained from cells grown in the presence of Tet. An increase of p53 transactivation activity by activated K-*ras* was also observed when transfected cells growing in the presence or absence of Tet were subjected to serum deprivation (0.1% serum, 24 h) before being collected for the CAT assay.

Induction of activated K-ras up-regulates bcl-2 but has no effect on bax and bcl-x

The studies described above showed that activated K-*ras* regulates both positive and negative modulators of the cell cycle such as the tumor suppressor gene product p53 and the E2F family of transcription factors. The net result of the seemingly opposing effects of activated K-*ras* induction was an increase in growth rate and acceleration of the cell cycle. Overexpression of both wild type p53 and E2F induces apoptosis, at least in some cell types (Wu and Levine, 1994). Preliminary studies in this laboratory indicated that induction of activated H-*ras* in MCF-7 cells renders cells more *resistant* to apoptotic cell death induced by treatment with DNA damaging agents such as cisplatinum (data not shown). Activated K-*ras* may

FBS (%)	10	10	0.1	0.1
Tet	+	-	+	-
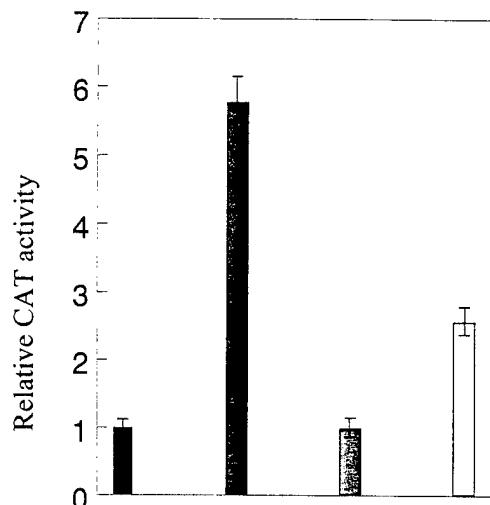
Figure 11 Effect of activated K-*ras* on transactivation activity of p53. The effect of activated K-*ras* on the p53 transactivation activity was measured as described in the legend for Figure 6 except that, instead of using pE2FCAT and pSVluc in the co-transfection experiments, cells were co-transfected with p53CONTKCAT (which contains the p53-responsive tk promoter driving the expression of the CAT reporter gene) and pSV2luc. The results represent the means±s.e. of relative CAT activities (after correction for transfection efficiency using luciferase activities) from three experiments

regulate certain negative modulators of apoptosis to compensate for the positive effect of p53 and E2F on apoptosis. Therefore, the effect of activated K-*ras* expression on bcl-2, bax and bcl-x, three well known regulators of apoptosis (Craig, 1995), was measured by Western blotting using specific antibodies against these proteins. As shown in Figure 12a, induction of activated K-*ras* by Tet withdrawal resulted in a significant increase in the level of bcl-2 expression (compare lanes 1 and 2). Induction of bcl-2 by activated K-*ras* was more profound when cells growing in the presence or absence of Tet were subjected to a 24 h serum deprivation (as described above) (compare lanes 3 and 4). As shown in Figure 12b and c, no obvious difference in the level of either bax or bcl-x expression was observed when cells were grown either in the presence or in the absence of Tet, with or without the serum deprivation step as described above. The up-regulation of bcl-2 may override the effect of increased expression of p53 and E2F on apoptosis, resulting in cells that are actually more resistant to apoptosis.

Discussion

A major objective of the current study was to examine the effect of activated K-*ras* on the cell cycle and to delineate the mechanism by which activated K-*ras* exerts this effect. A human breast carcinoma cell line MCF-7 was chosen as the model system for the current
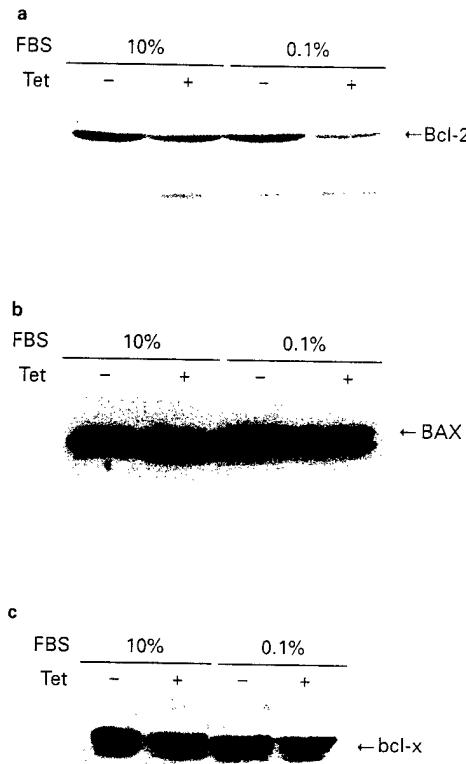
Figure 12 Effect of activated K-*ras* on expression of the *bcl-2* family members. M2TK4 cells were grown to mid-log phase, in the presence or absence of Tet, with or without a serum deprivation step, as described in the legend for Figure 3. Cells were collected by trypsinization and proteins from the total cellular extract were subjected to SDS–PAGE followed by Western blotting with specific polyclonal antibodies against *bcl-2* (**a**), *bax* (**b**) or *bcl-x* (**c**). Positions of these proteins are indicated by arrows. The figure represents one of two to four independent experiments

study for two major reasons: (1) it contains normal (wild type) *ras* genes and it has been widely used as a model (after transfection with activated *ras*) for studying the function of *ras* and (2) the cell line has been well characterized and it expresses wild type p53 (Negrini *et al.*, 1994) and pRb (Gottardis *et al.*, 1995).

It was shown in this paper that activated K-*ras* up-regulates cyclin D3 but not the other two members of the D-cyclin family (cyclins D1 and D2). To the best of our knowledge this is the first report showing that members of the D-type cyclin family are differentially regulated by activated *ras*. It is of interest to note that expression of cyclin D1, the only member of the D-type cyclin family considered to be an oncogene (Bates and Peters, 1995), is not affected by activated K-*ras* in this particular cell type. Our results suggest that cyclin D3, identified as a specific target for the activated K-*ras* in the current study, might also be classified as an oncogene. These data also suggest that each member of the D-type cyclin family may be independently regulated and may play, perhaps in a tissue specific manner, a distinct role in cell cycle regulation. A more detailed study on the mechanism by which cyclin D3 is regulated by activated K-*ras* awaits the availability of the cyclin D3 promoter sequences.

Three distinct members of the E2F family of transcription factors (E2F-1, E2F-4 and E2F-5), which display preferential binding to either pRb or the pRb-like proteins p107 and p130, respectively (Weinberg, 1996), were found to be up-regulated by activated K-*ras*, resulting in an overall increase in E2F transcriptional activity. In addition to up-regulating cyclins (e.g. A and E) and cyclin-associated kinase activities (e.g. CDK2 or CKD4) which would result in higher levels of 'free' E2F due to phosphorylation of the pRb family proteins, it is likely that activated K-*ras* regulates the transcription of E2Fs (i.e. the total E2F) by an unknown mechanism. Since this up-regulation by activated K-*ras* was found in all E2F forms, activated K-*ras* may regulate a common transcription factor essential for transcription for all E2F genes.

Several studies indicate that stabilization of the p53 protein might be a major mechanism for p53 accumulation in response to DNA damage (Canman *et al.*, 1994; Di Leonardo *et al.*, 1994). Although most studies show that MCF-7 cells contain wild type p53 (Takahashi *et al.*, 1992; Balcer-Kubiczek *et al.*, 1995), there have been some questions as to the functional status of the p53 tumor suppressor gene product present in MCF-7 cells (Casey *et al.*, 1991), the model cell line used in this study. Our data, obtained by use of a p53-responsive promoter construct suggests that the p53 protein up-regulated by activated K-*ras* in M2TK4 cells is transcriptionally active. In addition, activated K-*ras* induced both p21^{waf1} and Gadd 45, two well characterized p53 effectors whose expression is known to be controlled by p53 at the transcriptional level (Carrier *et al.*, 1994; Macleod *et al.*, 1995). Therefore MCF-7 cells used in this study most likely contain wild-type p53. Studies in this laboratory (Fan and Bertino, 1997) and elsewhere (Hiebert *et al.*, 1995) show that E2F-1 positively regulates p53 expression. As activated K-*ras* up-regulated three major forms of E2F, it is very likely that activated K-*ras* up-regulates p53 via a transcriptional mechanism, most probably via up-regulated E2F activity. A previous study (Negrini *et al.*, 1994) showed that MCF-7 cells, despite containing wild type p53, lack wild type p53 activity due to nuclear exclusion of p53. This suggests that activated K-*ras* may also regulate the activity of p53 by mechanisms other than gene regulation, such as post-translational modification.

The observation that p27^{Kip1} was down-regulated and p21^{waf1} was up-regulated by activated K-*ras* represents the first instance in which two CDK inhibitors are differentially regulated. Since expression of either p27^{Kip1} or p21^{Waf1} can result in cell cycle arrest (Toyoshima and Hunter, 1994; Wu *et al.*, 1996), their different regulation by activated K-*ras* cannot be perceived as a simple cell cycle effect. The same logic would be applicable to p53 and Gadd 45. Exactly how these CDK inhibitors are regulated by activated K-*ras* remains to be investigated.

Since both p53 and Gadd 45 have been implicated in DNA repair (Smith *et al.*, 1994; Gotz and Montenarh, 1996) and both of these molecules were up-regulated by activated K-*ras*, it is conceivable that cells containing activated K-*ras* would have a higher capacity for DNA repair. This would provide an explanation for activated *ras* mediated drug and radiation resistance

from previous studies in this laboratory (Fan *et al.*, 1997) and elsewhere (Levy *et al.*, 1994; Sklar, 1988). Up-regulation of *bcl-2*, but not *bax* or *bcl-x*, important regulators of apoptosis, by activated K-*ras* may explain why cells containing activated *ras* are generally more resistant to drug- or radiation-induced apoptosis, despite the up-regulation of both p53 and E2F by activated K-*ras*, shown previously to cooperate to induce apoptosis in Rat fibroblast cells (Wu and Levine, 1994). Up-regulation of *bcl-2* by K-*ras* also suggests that activated K-*ras* can overcome the inhibitory effect of p53 on *bcl-2* expression (Haldar *et al.*, 1994).

Although *ras* activation is generally believed to have a positive effect on cell cycle progression, several reports indicate that activated *ras* can induce cell cycle arrest in certain cell types (Pan *et al.*, 1994; Hirakawa and Ruley, 1988). Data presented in this paper provide strong evidence that activated K-*ras* exerts its effect on the cell cycle by modulating the expression of a diverse spectrum of cell cycle regulators such as the tumor suppressor protein p53 and the E2F family of transcription factors. The effect of activated *ras* on the cell cycle is characterized by two opposing functions: one is the negative regulatory function which is fulfilled by up-regulated p53 (accompanied by increased expression of p21^{waf1} and Gadd45) and the other is the positive regulatory function accomplished by increased expression of E2Fs and cyclins (A, E, and D3) and decreased expression of p27^{Kip1}. The net effect of activated K-*ras* on the cell cycle will be determined by a balance of these two opposing functions. When the negative cell cycle regulatory function of activated K-*ras* predominates over the positive effect, induction of activated K-*ras* may lead to slower cell growth or cell cycle arrest, and *vice versa*. Other factors such as the environment (such as growth factors) and cell types may also affect whether or not activated *ras* will have a positive or a negative effect on the cell cycle. Some investigators suggested that expression of activated ras at a level above certain threshold can result in growth arrest (Ricketts and Levinson, 1988).

Induction of wild type p53 activity (and subsequent induction of p21^{waf1} and Gadd 45) may be a major component of the negative cell cycle regulatory function of activated K-*ras*. This notion is supported by two previous studies showing that both dominant negative mutants of p53 (Hicks *et al.*, 1991) and the SV40 large T antigen (which binds to and inactivates p53) (Hirakawa and Ruley, 1988) were able to release *ras*-induced cell cycle arrest in a rat fibroblast cell line (REF52). The expected selective inhibition by mdm2 (believed to be an inhibitor of p53 (Finlay, 1993; Wu *et al.*, 1993)) of the negative regulatory function would explain the finding by Finlay (1993) that mdm2 together with activated *ras* can transform cells. Data presented in this paper also suggest that *ras* activation, as shown in this study, combined with findings from others regarding p53 loss, could lead to accelerated growth rates.

For chemotherapeutic purposes, targeting (and inhibition of) the positive cell cycle regulatory function of activated *ras* while leaving the other (negative regulatory) function intact may be a more effective and specific way to inhibit *ras*-mediated

tumorigenesis than targeting *ras* directly (Abrams *et al.*, 1996; Blume, 1993), especially if the tumor contains functional p53. Targeting *ras* directly may be complicated by the fact that *ras* is central to many cellular processes. It is reasonable to assume that in tumors containing both mutant p53 and activated *ras*, gene therapy with wild type p53 may not be an effective approach since the effects of p53 (which is inducible by *ras*) on the cell cycle and apoptosis may be offset by other effects of activated *ras* such as up-regulation of E2F and *bcl-2*.

Materials and methods

Chemicals and reagents

Tetracycline, bromodeoxyuridine (BrdU), propidium iodide, histone H1, bovine serum albumin (BSA), and acetyl CoA were obtained from Sigma. Geneticin (G418) was obtained from Life Technologies (Gaithersburg, MD). [γ-^{32}P]ATP (6000 Ci mmole), Econofluor-2, and [acetyl-1-^{14}C]-acetyl Coenzyme A were purchased from Dupont NEN. Media and sera for cell culture were purchased from Grand Island Biological Co., Grand Island, NY. Protein A Protein G Plus Agarose was obtained from Oncogene Science. Liposomes (DOTAP/DOPE) used for transient transfections were either purchased from Boehringer Mannheim (Indianapolis, IN) or made available by the Liposome Facility, Department of Medicine, Cornell University Medical College, NY. All other chemicals were reagent grade and from standard commercial sources.

Antibodies

Antibodies against K-*ras*, cyclin D1, cyclin D3, cyclin A, E2F-1, E2F-4, E2F-5, *bcl-2*, *bax*, *bcl-x*, p53, p21, p27, Gadd 45 and anti-cyclin A-agarose conjugates were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclin D2 antibody was obtained from NeoMarkers (Fremont, CA). Fluorescein isothiocyanate (FITC)-conjugated Anti-BrdU antibody was purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA). Polyclonal antibodies against human dihydrofolate reductase (DHFR) and thymidylate synthase (TS) were generous gifts from Dr Bruce Dolnick and Dr Frank Maley, respectively.

Cell line

The MCF-7 breast adenocarcinoma cell line was obtained from American Type Culture Collection and was maintained as monolayer cultures at 37°C in a 5% CO$_2$/95% air incubator in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Plasmids

The tetracycline inducible system plasmids pUHD10-3 and pUHD15-1 (Gossen and Bujard, 1992) were generous gifts from Dr Herman Bujard (Heidelberg, Germany). pAluc, which contains the cyclin A promoter linked to the firefly luciferase cDNA (Henglein *et al.*, 1994), was provided by Dr Berthold Henglein (Paris, France). pE2FCAT which contains the E2F-responsive promoter linked to the chloramphenicol transferase (CAT) cDNA (Buck *et al.*, 1995) was provided by Dr René Bernards (Amsterdam, The Netherlands). p53CONTKCAT which contains the p53-responsive tk promoter linked to the CAT cDNA (Yamato *et al.*, 1995) was obtained from Dr Nobuo Tsuchida (Tokyo, Japan). All plasmid DNAs were prepared from *E.*

coli strain DH5α and purified by ion exchange chromatography using the Plasmid Midi Kit from Qiagen (Chatsworth, CA).

Construction of an inducible vector system for an activated human K-ras

The tetracycline (Tet) responsive system (Gossen and Bujard, 1992), which is composed of two plasmids (pUHD10-3 and pUHD15-1), was used to construct the inducible vectors for controlled expression of activated K-ras. First, pUHD15-1 was modified to contain the selectable marker *neo*. This was accomplished by cloning the 1.1 kb tTA fragment from pUHD15-1 into the multiple cloning site of the plasmid pcDNA3 (Invitrogene, CA); the resulting plasmid is designated as pcdtTA. Then pUHD10-3 was modified to contain the cDNA sequences for human K-*ras* with a Gly^{12}→Val^{12} mutation (obtained from ATCC). The resulting plasmid is designated as pUHDkras2.

Cloning of MCF-7 cells

MCF-7 cells were cloned using the dilution method with a 96-well plate. Four different single clones were obtained and expanded into cell lines. One clone (#2) was used for transfection studies. DNA sequencing analysis showed that cells from all of these clones of MCF-7 contain a normal K-*ras* gene.

Stable transfection of the inducible activated K-ras construct into MCF-7 cells

The transfection of a single clone of MCF-7 cells with the Tet responsive system for the activated K-*ras* was accomplished by use of a two plasmid co-transfection protocol and a lipofection procedure (Stamatatos *et al.*, 1988). Cells were allowed to grow to ca. 50% confluency in a 100 mm petri dish in a medium containing 1 μg ml Tet. A total of 20 μg of the two plasmids (pUHDkras2 and pcdtTA) at a molar ratio of 10:5 were mixed with 70 μl of the lipofectant DOTAP in a total of 0.5 ml HBS (20 mM HEPES, pH 7.4 and 150 mM NaCl) and incubated at room temperature for 10 min. Fourteen ml of fresh growth medium containing Tet at 1 μg ml were added and, after removal of old medium from cells, the mixture was then added to cells and incubation continued for 24 h. Cells were split 1:5 and grown in a medium containing 1 μg/ml Tet. G418 selection began 24 h later at a concentration just high enough to kill 100% of the parental (untransfected) cells (800 μg/ml). About 2–5 weeks later, colonies were picked by the cloning cylinder technique and expanded into cell lines. Incorporation into these transfected cell lines of the inserted inducible activated K-*ras* cDNA was verified by use of PCR amplification using a CMV promoter-specific primer and a K-*ras* cDNA-specific primer.

Western blot analysis

MCF-7-K-*ras* cells were grown to mid-log phase in the presence of 1 μg/ml of Tet. After being washed extensively to remove extraneous Tet, the cells were collected by trypsinization and split equally into two culture flasks containing or lacking 1 μg/ml Tet, respectively. After 24 h incubation, the medium in each flask was changed with fresh media containing or lacking 1 μg/ml Tet. After an additional 48 h incubation, cells were harvested by trypsinization, washed with phosphate buffered saline (PBS), and solubilized with a buffer (50 mM NaCl, 20 mM Tris-HCl, pH 7.4) containing 2% (v v) Nonidet P-40 (NP-40), 0.5% (w v) sodium deoxycholate and 0.2% (w v) sodium dodecyl sulfate (SDS), plus a mixture of protease inhibitors (20 μg ml leupeptin, 30 μg ml aprotinin, 10 μg ml pepstatin A, 20 μg ml soybean trypsin inhibitor, 1 mM PMSF and 1 mM sodium orthovanadate). The extract was centrifuged at 60 000 *g* for 30 min to remove any insoluble cellular debris and the protein concentration was determined by the Bicinchoninic Acid (BCA) assay according to the manufacturer's instructions (Pierce). The cell extract (containing 150 μg of total protein) was mixed with equal volume of 2×SDS sample buffer (20% (v v) glycerol, 125 mM Tris-HCl, pH 6.8, 1% (v v) β-mercaptomethanol and 0.05% (w/v) bromophenol blue) and loaded onto a 12.5% polyacrylamide gel containing SDS (Laemmli, 1970). After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane and the latter was probed with an antibody (either a mouse monoclonal or a rabbit polyclonal), followed by a second antibody (either anti-mouse IgG or anti rabbit IgG) conjugated with peroxidase. The protein of interest was then visualized by treatment of the membrane with the Enhanced Chemiluminescence (ECL) reagents (Amersham) followed by exposure to a X-ray film.

Cell cycle analysis

Cells (5–10×10^6) growing exponentially at ca. 70% confluency in the presence or absence of Tet (1 μg/ml) were pulse-chase labeled with 10 μM BrdU for 3 h at 37°C in a CO$_2$ incubator. Cells were washed twice with PBS/BSA (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$ and 1% (w/v) BSA) and detached from the flask by trypsinization. After centrifugation at 500 *g* for 15 min at 4°C and resuspension in 100 μl of normal saline (0.9% (w v) NaCl) on ice, the cells were slowly added drop-wise to 5 ml pre-chilled (−20°C) 70% ethanol while maintaining a vortex. After incubation on ice for 30 min, the cell pellet was obtained by centrifugation, washed once with cold PBS, and resuspended by vortexing in 1 ml of 2N HCl containing 0.5% (v/v) Triton X-100. After another incubation for 30 min at room temperature, the cells were centrifuged at 500 *g* for 10 min and the pellet was resuspended in 1 ml of 0.1 M Na$_2$B$_4$O$_7$ (pH 8.5). Cells (1×10^6) were centrifuged and the pellet was resuspended in 50 μl PBS containing 0.5% (v v) Tween-20 and 1% (w/v) BSA. After addition of 20 μl of FITC conjugated anti-BrdU antibody and incubation for 30 min at room temperature, the cells were pelleted by centrifugation and resuspended in 1 ml PBS containing 5 μg/ml of propidium iodide (PI). The cell suspension was stored at 4°C overnight before analysis with a Becton Dickinson FACS flow cytometer. BrdU staining (FITC fluorescence) was plotted as a function of DNA content (PI fluorescence). Cells which were not labeled with BrdU but stained with FITC-BrdU antibody served as the control for background or non-specific anti-BrdU fluorescence so that percent of BrdU-positive (i.e. S phase) cells could be calculated.

Histone H1 kinase assays

The following was based on the procedures of Bortner and Rosenberg (1995), with modifications. Cells (1×10^7) growing exponentially at ca. 70% confluency in the presence or absence of Tet (1 μg/ml) were collected and the cell pellet was resuspended in 0.2 ml lysis buffer (100 mM Tris-HCl (pH 7.5) containing 300 mM NaCl, 2% (v/v) NP-40, 0.5% sodium deoxycholate, 0.2% (w v) SDS, 20 μg ml leupeptin, 30 μg ml aprotinin, 10 μg ml pepstatin A, 20 μg ml soybean trypsin inhibitor, 1 mM PMSF and 1 mM sodium orthovanadate). After incubation for 30 min on ice, the cell lysate was centrifuged at 15 000 r.p.m. (Eppendorf) for 15 min at 4°C. The protein concentration of the supernatant was determined by the BCA assay as described above. The cell extract containing 200 μg of

protein was treated with 50 μl of protein A protein G plus agarose on a rotator for 30 min at 4°C and the agarose beads were pelleted by centrifugation for 5 min at 1500 g (Eppendorf). The supernatant was then mixed with 16 μl of anti-cyclin A-agarose or with 3 μg of anti-cyclin D3 antibody plus 50 μl protein A/protein G plus-agarose. The mixture was rotated at 4°C for 16 h and the agarose beads were recovered by centrifugation, washed 4× with lysis buffer, and once with kinase buffer (50 mM HEPES (pH 7.5) containing 150 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol (DTT) and 2 mM ethylene glycosyl-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA)), and resuspended in 20 μl (for cyclin A) or 30 μl (for cyclin D3) of kinase buffer containing 100 μg/ml Histone H1, 10 μM ATP and 300 μCi/ml [γ-^{32}P]ATP. The kinase reaction was allowed to proceed for 1 h at 30°C and was stopped by addition of equal volume of 2 × SDS sample buffer. After being boiled for 3 min, the mixture was loaded onto a 12.5% polyacrylamide gel containing SDS and electrophoresis was performed at 10 mA at 4°C until the dye front reached 2 cm from the bottom of the gel. The gel was then dried and exposed to an X-ray film.

Transient transfections

MCF-7-K-ras cells were grown to mid-log phase in the presence of 1 μg/ml of Tet. After being washed extensively to remove extraneous Tet, the cells were collected by trypsinization and seeded at 1 × 10⁶ cells per plate into two separate sets of 100 cm culture dishes both of which contain 10 ml of RPMI 1640 medium supplemented with 10% serum; one group had 1 μg/ml of Tet added and the other had no Tet. After 24 h incubation at 37°C, the medium in each set of dishes was changed with fresh respective media containing or lacking 1 μg ml Tet and incubation continued for 5 h. Ten μg of total plasmid DNA consisting of the reporter plasmid and control plasmid were mixed with 0.5 ml serum-free RPMI 1640 medium and the resulting DNA mixture was incubated for 1 h at room temperature. Fifty μg of the lipofectant DOTAP/DOPE (1:1) were mixed with 0.5 ml serum-free RPMI 1640 medium and the resulting liposome mixture was incubated for 1 h at room temperature. The DNA mixture and the liposome mixture were combined and incubated for another 30 min at room temperature. The DNA/liposome mixture, after being diluted by addition of 6.5 ml of RPMI-1640 medium supplemented with 0.1% serum, with or without 1 μg ml of Tet, was added to cells in the culture plates whose medium had been removed. After incubation at 37°C for 5 h, 7.5 ml of RPMI 1640

medium supplemented with 20% serum, with or without Tet, was added to each dish and incubation was continued at 37°C for 16 h. Medium from each dish was replaced with fresh medium supplemented with 10% serum, with or without 1 μg ml of Tet, respectively and incubation was continued further at 37°C 48 h. After being washed twice with PBS, the transfected cells from each dish were recovered by use of 0.6 ml of the Reporter Lysis Buffer (Promega) and by scraping with a rubber policeman. The cell lysate was transferred to an Eppendorf tube and centrifuged to pellet large cell debris. The supernatant was stored at −70°C or used immediately for CAT and luciferase assays.

CAT and luciferase activity assays

The CAT activity of the cell lysate prepared from the transfected cells (see above) was measured by use of ¹⁴C-labeled acetyl Coenzyme A and chloramphenicol as substrates and a radio diffusion procedure according to manufacturer's instructions. Briefly, 50 μl of cell lysate was mixed with 0.2 ml 1.25 mM Chloramphenicol in 100 mM Tris buffer (pH 7.8) in a 7 ml glass scintillation vial. The reaction was initiated by adding 0.1 μCi [¹⁴C] Acetyl CoA and 22.5 μl 1 mM cold acetyl CoA. Five ml of Econofluor-2 was gently overlaid and the vials were incubated at room temperature. At timed intervals, the vials were counted using a Beckman Liquid Scintillation Counter. Relative CAT activity was calculated by comparison of the increase in counts over time. The luciferase activity was measured by use of the Luciferase Assay Reagent (Promega) according to the manufacturer's instructions. Briefly, 20 μl of cell lysate and 100 μl of the Luciferase Assay Reagent, both of which were pre-warmed to room temperature, were mixed and the relative light units produced for a period of 20 s were measured immediately on a luminometer (Monolight 2010, Analytical Luminescence Laboratory).

References

Abrams SI, Hand PH, Tsang KY and Schlom J. (1996). *Semin. Oncol.*, **23**, 118–134.

Adams PD and Kaelin WG Jr. (1995). *Semin. Cancer Biol.*, **6**, 99–108.

Bae I, Fan S, Bhatia K, Kohn KW, Fornace AJ Jr and O'Connor PM. (1995). *Cancer Res.*, **55**, 2387–2393.

Balcer-Kubiczek EK, Yin J, Lin K, Harrison GH, Abraham JM and Meltzer SJ. (1995). *Radiat. Res.*, **142**, 256–262.

Barbacid M. (1990). *Eur. J. Clin. Invest.*, **20**, 225–235.

Bates S and Peters G. (1995). *Semin. Cancer Biol.*, **6**, 73–82.

Beijersbergen RL, Kerkhoven RM, Zhu L, Carlee L, Voorhoeve PM and Bernards R. (1994). *Genes Dev.*, **8**, 2680–2690

Blake MC and Azizkhan JC. (1989). *Mol. Cell. Biol.*, **9**, 4994–5002.

Blume E. (1993). *J. Natl. Cancer Inst.*, **85**, 1542–1544.

Bortner DM and Rosenberg MP. (1995). *Cell Growth Differ.*, **6**, 1579–1589.

Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T and Hannon GJ. (1995). *Nature*, **377**, 552–557.

Buck V, Allen KE, Sorensen T, Bybee A, Hijmans EM, Voorhoeve PM, Bernards R and La Thangue NB. (1995). *Oncogene*, **11**, 31–38.

Canman CE, Chen CY, Lee MH and Kastan MB. (1994). *Cold Spring Harb. Symp. Quant. Biol.*, **59**, 277–286.

Carbonaro-Hall D, Williams R, Wu L, Warburton D, Zeichner-David M, MacDougall M, Tolo V and Hall F. (1993). *Oncogene*, **8**, 1649–1659.

Carrier F, Smith ML, Bae I, Kilpatrick KE, Lansing TJ, Chen CY, Engelstein M, Friend SH, Henner WD, Gilmer TM *et al.* (1994). *J. Biol. Chem.*, **269**, 32672–32677.

Casey G. Lo-Hsueh M. Lopez ME. Vogelstein B and Stanbridge EJ. (1991). *Oncogene.* **6**, 1791–1797.

Craig RW. (1995). *Semin. Cancer Biol.,* **6**, 35–43.

DeGregori J. Kowalik T and Nevins JR. (1995). *Mol. Cell. Biol.,* **15**, 4215–4224.

Devoto SH. Mudryj M. Pines J. Hunter T and Nevins JR. (1992). *Cell.* **68**, 167–176.

Di Leonardo A. Linke SP. Clarkin K and Wahl GM. (1994). *Genes Dev.,* **8**, 2540–2551.

el-Deiry WS. Tokino T. Velculescu VE. Levy DB. Parsons R. Trent JM. Lin D. Mercer WE. Kinzler KW and Vogelstein B. (1993). *Cell.* **75**, 817–825.

Fan J and Bertino JR. (1997). *Oncogene.* **14**, 1191–1200.

Fan J. Banerjee D. Stambrook PJ and Bertino JR. (1997). *Biochem. Pharmacol.,* in press.

Filmus J. Robles AI. Shi W. Wong MJ. Colombo LL and Conti CJ. (1994). *Oncogene.* **9**, 3627–3633.

Finlay CA. (1993). *Mol. Cell. Biol.,* **13**, 301–306.

Friedman PN. Chen X. Bargonetti J and Prives C. (1993). *Proc. Natl. Acad. Sci. USA.* **90**, 3319–3323.

Galaktionov K. Jessus C and Beach D. (1995). *Genes Dev.,* **9**, 1046–1058.

Gossen M and Bujard H. (1992). *Proc. Natl. Acad. Sci. USA.* **89**, 5547–5551.

Gottardis MM. Saceda M. Garcia-Morales P. Fung YK. Solomon H. Sholler PF. Lippman ME and Martin MB. (1995). *Endocrinology.* **136**, 5659–5665.

Gotz C and Montenarh M. (1996). *Rev. Physiol. Biochem. Pharmacol.,* **127**, 65–95.

Graham KA. Trent JM. Osborne CK. McGrath CM. Minden MD and Buick RN. (1986). *Breast Cancer Res. Treat.,* **8**, 29–34.

Haldar S. Negrini M. Monne M. Sabbioni S and Croce CM. (1994). *Cancer Res.,* **54**, 2095–2097.

Henglein B. Chenivesse X. Wang J. Eick D and Brechot C. (1994). *Proc. Natl. Acad. Sci. USA.* **91**, 5490–5494.

Hicks GG. Egan SE. Greenberg AH and Mowat M. (1991). *Mol. Cell. Biol.,* **11**, 1344–1352.

Hiebert SW. Packham G. Strom DK. Haffner R. Oren M. Zambetti G and Cleveland JL. (1995). *Mol. Cell. Biol.,* **15**, 6864–6874.

Hijmans EM. Voorhoeve PM. Beijersbergen RL. van 't Veer LJ and Bernards R. (1995). *Mol. Cell. Biol.,* **15**, 3082–3089.

Hirakawa T and Ruley HE. (1988). *Proc. Natl. Acad. Sci. USA,* **85**, 1519–1523.

Johnson DG. Schwarz JK. Cress WD and Nevins JR. (1993). *Nature.* **365**, 349–352.

Johnson LF. (1994). *J. Cell Biochem.,* **54**, 387–392.

Kato JY. Matsuoka M. Polyak K. Massague J and Sherr CJ. (1994). *Cell.* **79**, 487–496.

Khosravi-Far R and Der CJ. (1994). *Cancer Metastasis Rev.,* **13**, 67–89.

Laemmli UK. (1970). *Nature.* **227**, 680–685.

Levy E. Baroche C. Barret JM. Alapetite C. Salles B. Averbeck D and Moustacchi E. (1994). *Carcinogenesis.* **15**, 845–850.

Macleod KF. Sherry N. Hannon G. Beach D. Tokino T. Kinzler K. Vogelstein B and Jacks T. (1995). *Genes Dev.,* **9**, 935–944.

Mangues R and Pellicer A. (1992). *Semin. Cancer Biol.,* **3**, 229–239.

Marx J. (1994). *Science.* **264**, 1846.

Medema RH and Bos JL. (1993). *Crit. Rev. Oncog.,* **4**, 615–661.

Meek DW and Eckhart W. (1988). *Mol. Cell. Biol.,* **8**, 461–465.

Moll UM. LaQuaglia M. Benard J and Riou G. (1995). *Proc. Natl. Acad. Sci. USA.* **92**, 4407–4411.

Negrini M. Sabbioni S. Haldar S. Possati L. Castagnoli A. Corallini A. Barbanti-Brodano G and Croce CM. (1994). *Cancer Res.,* **54**, 1818–1824.

Nigg EA. (1995). *Bioessays.* **17**, 471–480.

Ohtsubo M. Theodoras AM. Schumacher J. Roberts JM and Pagano M. (1995). *Mol. Cell. Biol.,* **15**, 2612–2624.

Pagano M. Pepperkok R. Verde F. Ansorge W and Draetta G. (1992). *EMBO J.,* **11**, 961–971.

Pan BT. Chen CT and Lin SM. (1994). *J. Biol. Chem.,* **269**, 5968–5975.

Pines J. (1995). *Semin. Cancer Biol.,* **6**, 63–72.

Pines J and Hunter T. (1992). *Ciba. Found. Symp.,* **170**, 187–196: *discussion* 196–2.

Polyak K. Kato JY. Solomon MJ. Sherr CJ. Massague J. Roberts JM and Koff A. (1994). *Genes Dev.,* **8**, 9–22.

Ricketts MH and Levinson AD. (1988). *Mol. Cell. Biol.,* **8**, 1460–1468.

Rodenhuis S. (1992). *Semin. Cancer Biol.,* **3**, 241–247.

Rosenberg AR. Zindy F. Le Deist F. Mouly H. Metezeau P. Brechot C and Lamas E. (1995). *Oncogene.* **10**, 1501–1509.

Sardet C. Vidal M. Cobrinik D. Geng Y. Onufryk C. Chen A and Weinberg RA. (1995). *Proc. Natl. Acad. Sci. USA.* **92**, 2403–2407.

Sherr CJ. (1995). *Trends Biochem. Sci.,* **20**, 187–190.

Sherr CJ and Roberts JM. (1995). *Genes Dev.,* **9**, 1149–1163.

Sklar MD. (1988). *Science.* **239**, 645–647.

Slansky JE. Li Y. Kaelin WG and Farnham PJ. (1993). *Mol. Cell. Biol.,* **13**, 1610–1618.

Smith ML. Chen IT. Zhan Q. Bae I. Chen CY. Gilmer TM. Kastan MB. O'Connor PM and Fornace AJ Jr. (1994). *Science.* **266**, 1376–1380.

Stacey DW and Kung H-F. (1984). *Nature.* **310**, 508–511.

Stamatatos L. Leventis R. Zuckermann MJ and Silvius JR. (1988). *Biochemistry.* **27**, 3917–3925.

Takahashi K. Suzuki K. Uehara Y and Ono T. (1992). *Jpn. J. Cancer Res.,* **83**, 358–365.

Toyoshima H and Hunter T. (1994). *Cell.* **78**, 67–74.

Walker DH and Maller JL. (1991). *Nature.* **354**, 314–317.

Weinberg RA. (1995). *Cell.* **81**, 323–330.

Weinberg RA. (1996). *Cell.* **85**, 457–459: 242.

Westwick JK. Cox AD. Der CJ. Cobb MH. Hibi M. Karin M and Brenner DA. (1994). *Proc. Natl. Acad. Sci. USA.* **91**, 6030–6034.

Winston JT. Coats SR. Wang YZ and Pledger WJ. (1996). *Oncogene.* **12**, 127–134.

Wu CL. Zukerberg LR. Ngwu C. Harlow E and Lees JA. (1995). *Mol. Cell. Biol.,* **15**, 2536–2546.

Wu H. Wade M. Krall L. Grisham J. Xiong Y and Van Dyke T. (1996). *Genes Dev.,* **10**, 245–260.

Wu X. Bayle JH. Olson D and Levine AJ. (1993). *Genes Dev.,* **7**, 1126–1132.

Wu X and Levine AJ. (1994). *Proc. Natl. Acad. Sci. USA.* **91**, 3602–3606.

Xiong Y. Hannon GJ. Zhang H. Casso D. Kobayashi R and Beach D. (1993). *Nature.* **366**, 701–704.

Xu M. Sheppard KA. Peng CY. Yee AS and Piwnica-Worms H. (1994). *Mol. Cell. Biol.,* **14**, 8420–8431.

Yamato K. Yamamoto M. Hirano Y and Tsuchida N. (1995). *Oncogene.* **11**, 1–6.

Yang BS. Hauser CA. Henkel G. Colman MS. Van Beveren C. Stacey KJ. Hume DA. Maki RA and Ostrowski MC. (1996). *Mol. Cell. Biol.,* **16**, 538–547.

Zindy F. Lamas E. Chenivesse X. Sobczak J. Wang J. Fesquet D. Henglein B and Brechot C. (1992). *Biochem. Biophys. Res. Commun.,* **182**, 1144–1154.

Tetracycline-Controlled Gene Expression System Achieves High-Level and Quantitative Control of Gene Expression

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The tetracycline-controlled gene expression system utilizes the control elements of the tetracycline resistance operon encoded in Tn10 of *Escherichia coli* to control gene expression in eukaryotic cells. Here we demonstrate the quantitative control of the expression of the luciferase gene, dihydrofolate reductase gene, and *bcl-2* gene in HeLa S3 or Chinese hamster ovary AA8 cells using the tetracycline-controlled gene expression system. Regardless of the host cell lines or the genes being expressed, there is a common range of tetracycline concentration within which the expression of genes is most sensitively regulated. In addition, the maximal gene expression level of the tetracycline-controlled gene expression system is higher than that of the wild-type CMV promoter/enhancer-driven system. Nonetheless, careful selection of stably transfected clones is necessary to achieve the optimally regulated gene expression using this system. © 1996 Academic Press, Inc.

The tetracycline-controlled gene expression system has gained increasing popularity since its invention (1, 2). To employ this system to stably express any gene of interest, the host cell lines expressing the tetracycline-controlled transactivator (tTA)[1] must first be generated. The tTA is composed of the C terminal 127 amino acids of the herpes simplex virus VP16 fused with the prokaryotic tet repressor protein (tetR). Therefore, the first step is to stably transfect the plasmid pUHD15–1, which contains the tTA gene, into the desired host cell lines. Next, the cDNA encoding the gene of interest is inserted into the plasmid pUHD10–3 at a position

downstream of the promoter PhCMV*-1, which is composed of seven repeats of the tetR binding sequence (tetO2) followed by the hCMV minimal promoter. After this plasmid is stably transfected into the tTA-expressing host cells, tTA can bind to the tetO2 sequences, bringing VP16 to the proximity of the hCMV minimal promoter, thus activating transcription. However, if tetracycline is added to the cell culture media, tetracycline binds to tTA, causing it to dissociate from PhCMV*-1, thus inhibiting transcription.

The tetracycline-controlled gene expression system has been successfully employed in a number of studies (3–5). Nonetheless, some researchers experienced difficulty when trying to express their genes of interest in their favorite cell lines. Here we share some of our experience of using the tetracycline-controlled gene expression system, focusing on four aspects: (i) Careful selection of the clones expressing tTA and the subsequent selection of clones expressing the target gene are necessary to achieve the desired control of target gene expression. (ii) The level of target gene expression can be quantitatively controlled by controlling the concentration of tetracycline. The range of tetracycline concentration that can achieve the most sensitive control of gene expression remains constant for different cell lines and different genes. This system will be an invaluable tool for "quantitative intracellular biochemistry" research. (iii) The maximal expression level and the basal expression level that can be achieved with this system may be different for different cell types. However, we were able to achieve tetracycline-regulated gene expression in all four cell types that we used. (iv) When optimally selected stable tTA-expressing cell lines were used, the maximal expression level obtained in the tetracycline-controlled gene expression system is higher than that of the CMV promoter/enhancer-driven system. Therefore, the tetracycline-controlled gene expression system can replace

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[1] Abbreviations used: tTA, tetracycline-controlled transactivator; tetR, tet repressor protein; DHFR, dihydrofolate reductase.

the traditional CMV promoter/enhancer-driven systems in most applications.

MATERIAL AND METHODS

Plasmids

pUHD15–1 contains the tTA coding sequence driven by the hCMV promoter/enhancer. pUHD10–3 contains the PhCMV*-1 followed by a multiple cloning sequence. pUHC13–3 has a luciferase cDNA driven by PhCMV*-1 (1). All three plasmids were generous gifts from H. Bujard.

To generate pDHFRUHD, the HhaI to BglII fragment of the dihydrofolate reductase (DHFR) cDNA clone DHFR11 (6) was inserted into the BamHI and SmaI restriction sites of pUC18 to generate DHFR/pUC18. Then the EcoRI to XbaI fragment of DHFR/pUC18 was cloned into the EcoRI and XbaI restriction sites of pUHD10–3 to produce pDHFRUHD. To generate pBcl2UHD, the 845-bp EcoRI to XhoI fragment, which contains the full coding sequence of bcl-2 cDNA, was excised from pBluebcl-2 and cloned into the EcoRI and XbaI sites of pUHD10–3 (5). The noncohesive ends were made blunt with T4 DNA polymerase prior to ligation.

To generate pCMV-Luc, the 1118-bp PvuI to SalI fragment of pUHC13–3 was replaced with the 1524-bp PvuI to XhoI fragment from pcDNA3 (Invitrogen). The resulting pCMV-Luc is identical to pUHC13–3 except that the promoter PhCMV*-1 is replaced by the wild-type CMV promoter in pcDNA3.

Construction of Cell Lines and Luciferase Assays

Human epithelioid carcinoma cell HeLa S3, Chinese hamster ovary cell AA8, human breast adenocarcinoma cell MCF-7, and mouse embryo cell NIH3T3 that stably express tTA were produced as follows. Forty micrograms of pHUD15–1 was linearized with ScaI and cotransfected with 2 µg pSV2neo into the target cells. All transfections described in this report were performed by electroporation using a Bio-Rad gene pulser at 220 V, 950 µF in 0.4-cm cuvettes in α-MEM or DMEM plus 10% fetal bovine serum (GIBCO-BRL). Cells resistant to 400 µg/ml G418 were cloned and tested for tTA expression by an assay for the transient expression of the luciferase gene in pUHC13–3. In this assay, 10 µg pUHC13–3 was electroporated into 1×10^6 cells. Cells were split into two 10- or 6-cm dishes, and tetracycline was added into one dish to 2 µg/ml. Luciferase assays were performed 48 h after transfection with Promega luciferase assay system using 10 µl cell lysate and 50 µl luciferase substrate and measured with a Monolight 2010 luminometer (Analytical Luminescence Laboratory) (Figs. 1 and 2) or a Turner TD-20e luminometer (Figs. 3 and 4). Protein concentration was determined with the Bio-Rad protein assay kit using 5 µl cell lysate,

150 µl protein assay solution, and 600 µl water. The luminometer readings in the luciferase assays divided by the optical density readings at 600 nm in the protein assay were plotted as the luciferase activity.

The stable transfection of pUHC13–3, pDHFRUHD, or pBcl2UHD into tTA-expressing HeLa S3 or AA8 cells was performed by electroporation using the same conditions described above. Forty micrograms of ScaI-linearized pDHFRUHD or pBcl2UHD was electroporated into the appropriate host cells. Two micrograms of the BamHI and ClaI fragment of pDR2, which contains the hygromycin-resistant gene driven by the HSV TK promoter, was also included in the electroporation to provide the hygromycin selection marker (5). Cells were selected with 200 µg/ml hygromycin B (Calbiochem). The resulting colonies were cloned and further analyzed.

Northern and Western Analysis

For Northern analysis, 20 µg total RNA was loaded per lane on a 1% denaturing agarose/formaldehyde gel. After electrophoresis, the gel was blotted and hybridized using standard procedures (7). Western blot analysis was performed as described (5, 8), using both a human Bcl-2 specific mAb (DAKO, 1:1000 dilution) and a human cycline B1-specific mAb (PharMingen, 1:1000 dilution) mixed together to detect both proteins simultaneously.

RESULTS AND DISCUSSIONS

Careful Selection of the tTA-Expressing Clones Is Necessary for Optimal Results

After transfection with pUHD15–1, independent G418-resistant clones were analyzed using the transient luciferase expression assay. A wide range of clonal variation of the efficacy of tetracycline-controlled gene expression among different tTA-expressing clones was observed. For example, of the 12 tTA-expressing Hela S3 cell clones that we analyzed in one electroporation experiment, the effectiveness of tetracycline regulation of luciferase gene expression varied from 20- to 1100-fold (Fig. 1A). This variation is mainly due to clonal difference of the maximal expression level. The maximal expression level (the expression level in the absence of tetracycline) of these clones varied by 455-fold, whereas the basal expression level (the expression level in 2 µg/ml tetracycline) varied by less than 9-fold (data not shown). This clonal variance is most likely caused by the difference of tTA concentration in each clone, as will be discussed later. Therefore, many clones need to be analyzed to identify the optimal tTA-expressing clone for subsequent transfection of the target gene. This is one reason we do not recommend cotransfecting pUHD15–1 and the target gene. Clone 7 (also named H7 or HeTe7; Fig. 1A) was selected as our opti-
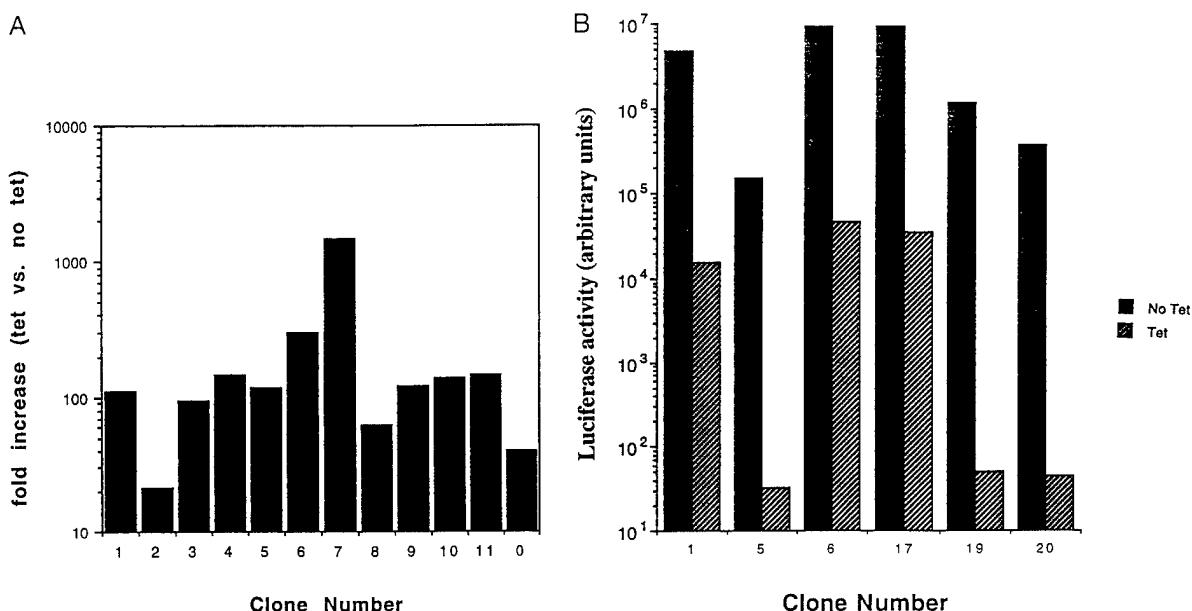
FIG. 1. (A) Different clones of tTA-expressing HeLa S3 cells achieved different levels of tetracycline-regulated gene expression. H7 cells were transiently transfected with 10 μg pUHC13–3. The luciferase activity in the absence of tetracycline divided by that in 2 μg/ml tetracycline is plotted as fold increase. (B) Luciferase activities of tTA-expressing AA8 cells (clone A17) stably transfected with pUHC13–3. Different clones can have either high background and high maximal expression (clones 1, 6, and 17) or low background and low maximal expression (clones 5, 19, and 20). All cell lines were grown for 2 days in the absence of tetracycline (no tet) or in 2 μg /ml tetracycline (tet) before being assayed.

mal tTA-expressing Hela S3 clone. This clone had the highest maximal luciferase expression and a low basal luciferase expression in the transient luciferase assay.

We have not been successful in directly quantifying tTA concentration by simple techniques, such as Western blot analysis. However, we have obtained one subclone of H7 which amplified the tTA gene by more than 10-fold (Yin *et al.*, manuscript in preparation). In the absence of tetracycline, the expression of the transiently transfected PhCMV*-1-driven luciferase gene (pUHC13–3) or stably transfected PhCMV*-1-driven dihydrofolate reductase gene (pDHFRUHD) was up to 100-fold higher in this subclone than in the parental H7 cells. The basal expression level (in 2 μg/ml tetracycline) is not significantly different between this subclone and H7 (Yin *et al.*, manuscript in preparation). This observation provides indirect evidence that tTA concentration is critical for the effectiveness of the tetracycline-controlled gene expression system. This also provides an explanation why the expression efficiency of this system, in selected tTA-expressing clones, is higher than that of the wild-type CMV promoter/enhancer-driven system.

Clones Expressing the Target Genes Also Need to Be Carefully Selected for the Desired Results

After the PhCMV*-1-driven target gene is integrated into a tTA-expressing cell line, different clones also behave quite differently. For example, we cotransfected pUHC13–3 and a hygromycin-resistant gene into a tTA-expressing AA8 cell line (clone A17) and found that the 37 hygromycin-resistant clones tested fell into three distinct categories: 4 clones did not express the luciferase gene; 14 clones expressed the luciferase gene with an extremely low background in the presence of 2 μg/ml tetracycline and >10,000-fold higher in the absence of tetracycline (represented by clones 5, 19, and 20 in Fig. 1B), and the other 19 clones had approximately 100-fold higher maximal luciferase expression than the previous 14 clones, but the basal expression level was about 1,000-fold higher (represented by clones 1, 16, and 17 in Fig. 1B). Similar results were obtained when pDHFRUHD was transfected into AA8 cells (data not shown). We speculate that such differences result from the pattern of integration of the PhCMV*-1-driven target gene. Since PhCMV*-1 only lacks an enhancer element to activate transcription, if the target gene integrates near an endogenous enhancer element in the genome, this enhancer can activate target gene transcription even in the presence of tetracycline, resulting in a high basal expression level. When there is no tetracycline, tTA will act synergistically with the endogenous enhancer to induce a extremely high expression of the target gene. However, if the target gene integrates into a "silent" stretch of the genome that is not under the influence of an enhancer, the basal expression level will be very low.

Therefore, it is necessary to carefully select clones that best suit the intended purpose, whether it is high expression or low background.

We speculate that since different cell lines have a different composition of transcription activator proteins, and probably a different restraint for plasmid integration, they will behave differently when used as hosts for the tetracycline-controlled expression system. For example, in cell lines that have many transcription activator proteins, a larger fraction of endogenous enhancer elements are active. Therefore, the integration site of the target gene is more likely to be under the influence of an active enhancer, and more clones need to be screened to identify one which has low basal expression. For cell lines that have fewer transcription activator proteins, more clones may have low background expression but also relatively low maximal expression.

Since cotransfection tends to produce tandem cointegration, we do not recommend the strategy of cotransfecting pUHD15-1 and the plasmid carrying the PhCMV*-1-driven target gene, although this strategy may be useful if the intended effect is to generate cell lines with high background and superhigh maximal expression. With this strategy, the CMV enhancer on pUHD15-1 is very likely to be placed near PhCMV*-1. Therefore, most of the clones will have high background and superhigh maximal expression. Low-background clones only occur very rarely, when two independent integrations happen and the target gene is integrated in a region of the genome not influenced by endogenous enhancers. For the same reason, we believe that the drug selection markers generally should not be cloned onto pUHD10-3, and the promoter for the drug selection marker should have a low enhancer activity, such as the TK promoter. Bujard et al. have independently reached the same conclusion (H. Bujard and M. Gossen, personal communication).

Theoretically, the copy number of the integrated PhCMV*-1-driven target gene could be another factor that influences the target gene expression pattern. However, Southern blot analysis revealed that our experimental procedure only resulted in single-copy or occasionally two-copy integration. There seems to be no apparent correlation between the copy number of the target gene and the expression pattern in our experiment (data not shown). However, we cannot rule out copy number as an influence of gene expression pattern if other methods are used to generate stable transfectants that produce multiple-copy integration.

Tetracycline Quantitatively Controls Gene Expression

We have stably expressed luciferase, Bcl-2, and DHFR genes with the tetracycline-controlled expression system in tTA-expressing CHO AA8 cells (clone A17) and HeLa S3 cells (clone H7). Figure 2A shows the expression of the luciferase gene in A17 cells at different tetracycline concentrations. The two clones shown are clones 17 and 19 of Fig. 1B. The result of clone 19 is very similar to that of Gossen and Bujard's original result using HeLa cells (clone X1) (1). Although clone 17 had a higher background and higher maximal expression level, the range of tetracycline concentration that achieved the most sensitive control of gene expression remained between 0.0001 and 0.1 μg/ml. This observation is consistent with the interpretation that the luciferase gene in clone 17 is under the influence of an endogenous enhancer which functions independently to but synergistically with tTA. Figure 2B shows a Northern blot of the mRNA for the mouse DHFR gene in pDHFRUHD stably expressed in the A17 cells. Figure 2C shows a Western blot of the Bcl-2 protein expressed from stably integrated pBcl2UHD in the H7 cells (5). It is evident that the regulation of gene expression by tetracycline with this system is quantitative, and the same range of tetracycline concentration (0.0001–0.1 μg/ml) achieves the most sensitive control of gene expression. This system offers the opportunity to perform intracellularly the quantitative biochemical analysis similar to the classical enzymology assays. It also allows the analysis of the same cells expressing different amounts of a particular protein, eliminating the possibility of clone heterogeneity which can complicate the interpretation of experimental results. These two benefits have allowed researchers to reach conclusions that could not be reached with certainty by traditional gene expression strategies (5). Of course, the tetracycline-controlled gene expression system also allows the stable expression of toxic genes because of its ability to tightly repress the expression of the target gene in transfected clones (Fig. 2, Ref. 3).

Different Cell Types Have Somewhat Different Behavior with the Tetracycline-Controlled Expression System

We have expressed tTA in four different cell lines. Figure 3 shows the efficacy of the tetracycline-controlled expression system in these cell lines, as measured by the transient luciferase assay. The HeLa S3, AA8, MCF-7, and NIH3T3 cells shown were selected from 12, 23, 16, and 95 tTA-expressing clones, respectively, because they showed the highest maximal luciferase expression when pUHC13-3 was transfected in the transient transfection assay. Although it was fairly easy to find HeLa and MCF-7 cells with high-level induced and low-level background expression, it was more difficult to do so with NIH3T3 cells. We do not have a explanation for such a phenomenon. Nevertheless, this observation may be valuable information for researcher interested in applying this system in different cell lines.

FIG. 2. Tetracycline quantitatively regulates gene expression. In each experiment, cells were cultured 48 h in the indicated concentration of tetracycline before being assayed. (A) PhCMV*-1-driven luciferase gene expression in tTA-expressing AA8 cells (clones 17 and 19 of Fig. 1B). (B) Northern blot of PhCMV*-1-driven mouse DHFR gene expression in tTA-expressing AA8 cells (clones 13, 18, and 26). No RNA was loaded in the 0.002 μg/ml lane for clone 13. Equal loading was confirmed by ethidium bromide staining of the gel (data not shown). (C) Western blot staining of PhCMV*-1-driven *bcl-2* gene expression in HeLa S3 cells.

FIG. 3. Tetracycline-controlled gene expression system functions in AA8, HeLa S3, NIH 3T3, and MCF7 cells. The tTA-expressing cell clones shown are A17 (AA8), H7 (HeLa S3), 92 (NIH 3T3), and 5I (MCF7). Error bars are SD for two experiments.

The Maximally Induced Expression Level of the Tetracycline-Controlled Expression System Is Very High

In the assays shown in Fig. 4, the luciferase activity of H7 cells transfected with 10 μg of pCMV-Luc or pUHC13-3 was compared. The maximal expression level of the tetracycline-controlled gene expression system is approximately 35 times stronger than that of the CMV promoter/enhancer-driven system. We propose two speculations to explain this high transcription efficiency. First, the tetR–tetO2 binding is a very strong protein–DNA interaction (9). Second, the H7 cells, and the other tTA-expressing cells that we used to transfect the target genes, were selected from many tTA-expressing clones for maximal tTA activity. Based on the results in Fig. 4, and the fact that in the absence of tetracycline, clone H7 expressed the PhCMV*-1-driven luciferase gene 455 times stronger than the least active tTA-expressing HeLa S3 clone among the 12 clones analyzed, we can infer that PhCMV*-1 is approximately 7% to 35 times as active as the wild-type CMV promoter in various tTA-expressing HeLa S3 clones. Therefore, selection of the optimal tTA-expressing clones is very important for achieving the high expression activity of the tetracycline-controlled gene expression system. Gossen observed that when pUHD15-1 and pUHC13-3 were cotransfected into HeLa cells, the resulting luciferase activity was about twice that of pCMV-Luc, indicating that PhCMV*-1 is slightly stronger than the CMV promoter/enhancer even when tTA concentration is not optimized (10).

Although the repressed expression level of the tetracycline-controlled gene expression system in Fig. 4 ap-

pears to be only about 10-fold lower than that of pCMV-Luc, such a basal expression is an artifact of the transient transfection assay. For example, with this transient expression assay, the A17 cells have $6.0 \pm 1.5 \times 10^3$ basal and $6.1 \pm 3.5 \times 10^5$ maximal luciferase activity (mean $\pm$ SD for three experiments) when measured at the scale of Figs. 1B and 2A. The basal and maximal expressions of the best stable clones (clones 5, 19, and 20) were approximately 20 and $>10^6$, respectively (Figs. 1B and 2A). Therefore, the tetracycline-controlled gene expression system tends to show much higher background and much lower maximal expression in transient assays than in stable clones.

Our results indicate that tTA concentration probably has a strong effect on the maximal expression of PhCMV*-1-driven genes, but does not affect the basal expression level significantly. The background expression of the target gene can be reduced by analyzing a large number of stable transfectants. The maximal expression of the target is very high in carefully selected tTA-expressing host cells. These observations theoretically have some implication on the recently published strategy of the "autoregulatory" system (11). In this system, the tTA gene is also under the control of PhCMV*-1, generating an autoregulatory loop. Such a system puts tTA under the control of PhCMV*-1, which is a stronger promoter than the CMV promoter when and only when there is a sufficient amount of tTA. Therefore, tTA expression theoretically could be higher in this system, but only if the autoregulation reaches equilibrium at a high tTA concentration. With tTA concentration itself being a variable, and with no knowledge of what actually determines the equilibrium status in this autoregulatory system, it is impossible to predict the reproducibility of this autoregulatory strategy for different cell lines or even for different
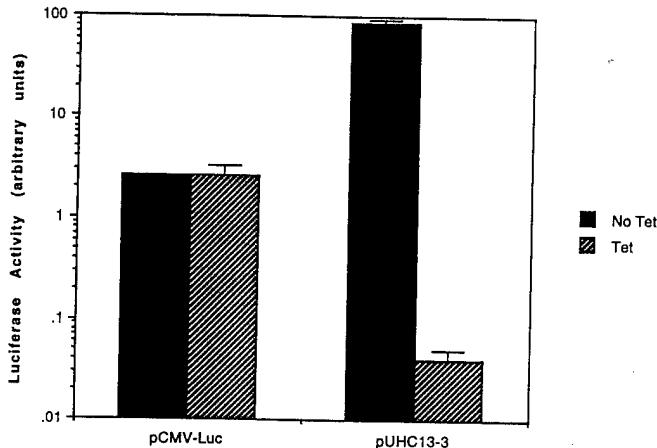
FIG. 4. H7 cells transiently transfected with 10 μg pUHC13-3 have a stronger luciferase activity than H7 cells transfected with 10 μg pCMV-Luc.

experiments in the same cell line. In addition, it is not known whether the autoregulatory system can achieve consistent quantitative control of gene expression. Furthermore, because tTA concentration does not affect the basal expression level significantly, the autoregulatory strategy in theory will not reduce the background expression. The effectiveness of the autoregulatory strategy can only be evaluated after sufficient amounts of data are collected. Nonetheless, we feel that the improvement of the autoregulatory strategy, even if proven to be consistent, is incremental and not necessary for most research involving cultured mammalian cells. With carefully chosen tTA expression cells, the original Gossen and Bujard system achieves much higher expression than the CMV promoter/enhancer system, which is sufficient for almost all applications.

We predict that when tTA-expressing cell lines become common laboratory cell lines, the tetracycline-controlled gene expression system can replace the CMV promoter/enhancer-driven expression system and many other unregulated gene expression systems in many applications. The tetracycline-controlled expression system expresses genes at high levels in normal media without tetracycline, but has the additional capability of quantitatively suppressing the expression by the addition of tetracycline. With a collection of tTA-expressing cell lines, it is as easy to express genes with the tetracycline-controlled system as it is with any other current system.

REFERENCES

1. Gossen, M., and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5547–5551.
2. Gatz, C., and Quail, P. H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1394–1397.
3. Resnitzky, D., Gossen, M., Bujard, H., and Reed, S. I. (1994) *Mol. Cell. Biol.* **14**, 1669–1679.
4. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) *J. Cell Biol.* **127**, 915–934.
5. Yin, D. X., and Schimke. R. T. (1995) *Cancer Res.* **55**, 4922–4928.
6. Chang, A. C. Y., Nunberg, J. H., Kaufman, R. J., Erlich, H. A., Schimke, R. T., and Cohen, S. N. (1978) *Nature* **275**, 617–621.
7. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
8. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, p. 479, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
9. Hinrichs, W., Kisker, C., Duvel, M., Muller, A., Tovar, K., Hillen, W., and Saenger, W. (1994) *Science* **264**, 418–420.
10. Gossen, M. (1993) Ph.D. Thesis, University of Heidelberg, Germany.
11. Shockett, P., Difilippantonio, M., Hellman, N., and Schatz, D. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 6522–6526.

CELLULAR AND MOLECULAR BIOLOGY ™

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THE ROLE OF INTEGRINS AND EXTRACELLULAR MATRIX IN ANCHORAGE-INDEPENDENT GROWTH OF A MAMMARY CARCINOMA CELL LINE

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Abstract - Anchorage-independent growth is a property of malignant cells. Extracellular matrix proteins are present in tumor spheroids but their function is not clearly defined. In this paper we show that a murine mammary carcinoma cell line, SP1, which expresses the fibronectin receptor $\alpha_5\beta_1$ requires fibronectin for anchorage-independent growth in soft agar. Growth factors (hepatocyte growth factor and transforming growth factor-β) also promote SP1 colony growth. In contrast, collagen types I and IV have an inhibitory effect on SP1 colony growth. A clone isolated from SP1 cells which expresses the collagen/laminin receptor $\alpha_2\beta_1$ as well as the fibronectin receptor $\alpha_5\beta_1$, demonstrates increased colony formation in the presence of fibronectin and collagen. These data suggest a role for both the $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrin receptors in the regulation of anchorage-independent growth of mammary carcinoma cells.

Key words: Fibronectin, collagen I, anchorage-independent growth, growth factors, breast cancer

INTRODUCTION

Tumor cells exhibit a number of different characteristics compared to their normal cell counterparts. The more prominent of these phenotypic changes are unlimited cell division potential, rapid proliferation, and loss of adhesive requirement for survival and growth under anchorage-independent conditions. It is well known that millions of cells can disseminate from a primary lesion into the blood stream, however, few cells actually grow as metastatic nodules in other organs (Hart and Saini, 1992;

Poste and Fidler, 1980). Folkman and Moscona (1978) have shown that normal cells need to adhere and spread in order to grow in response to growth factors; however most transformed cells have lost the cell adhesion and spreading requirement. Thus tumor spheroids cultured *in vitro* are considered to resemble *in vivo* metastatic nodules and are more representative of the tumorigenic phenotype than cells in 2-dimensional culture (Freedman and Shin, 1974). Before the characterization of integrins, several reports demonstrated the presence of extracellular matrix (ECM) proteins in tumor spheroids (De Pauw-Gillet *et al.*, 1988; Glimelius *et al.*, 1988); however, the role of integrins and interactions with growth factors in growth regulation under anchorage-independent conditions has not been thoroughly investigated.

Abbreviations: **Coll I**: collagen type I; **ECM**: extracellular matrix; **FBS**: fetal bovine serum; **FN**: fibronectin; **HGF**: hepatocyte growth factor; **LM**: laminin; **TGF-β**: transforming growth factor-β; **VN**: vitronectin

Integrins are transmembrane heterodimers consisting of an α and β chain that mediate adhesion to the ECM and some cell-cell interactions (Albelda and Buck, 1990; Giancotti and Mainiero, 1994; Hynes, 1992). Following integrin ligation, both mechanical and biochemical signals are transmitted to the nucleus, and thereby regulate such functions as cell adhesion and spreading, migration, invasion and matrix remodelling (Akiyama *et al.*, 1989; Wu *et al.*, 1993). Although there is a general reduction of integrin receptors on transformed cells, the pattern of expression is variable in each tumor and may vary considerably within a single tumor (Miettinen *et al.*, 1993).

A reduction of $\alpha_2\beta_1$ integrin expression on some human mammary carcinomas was reported by Pignatelli *et al.* (1991), although D'Ardenne *et al.* (1991) found a uniform positive staining for $\alpha_2\beta_1$ in both benign and malignant breast epithelium. Recently, Zutter *et al.* (1995) found that ectopic expression of the $\alpha_2\beta_1$ collagen receptor in a murine mammary carcinoma cell line, results in increased differentiation and abrogates the malignant phenotype of these cells. Furthermore, Hangan *et al.* (1996) showed that expression of a transfected α_2 gene in a rhabdomyosarcoma inhibits the ability of these cells to migrate through liver tissue, most likely due to increased adhesion to basement membrane, and abrogates metastasis formation. Together, these findings raise the possibility that $\alpha_2\beta_1$ may play a negative regulatory role in tumor progression of some cancer cells.

Some non-metastatic mammary tumors express $\alpha_5\beta_1$ integrin, while expression on metastatic tumors was generally reduced (Peña *et al.*, 1994). Additionally, over-expression of a transfected α_5 integrin gene in CHO cells inhibits anchorage-independent growth and tumorigenicity (Giancotti and Ruoslahti, 1990). However, other cells require low levels of $\alpha_5\beta_1$ integrin for proliferation in response to growth factors (Symington, 1995). Thus the amount of α_5 integrin expressed is critical in determining the affect of fibronectin adhesion via this receptor on tumor cell function. This apparent

paradox may be resolved with the recent finding that expression of $\alpha_5\beta_1$ without ligation induces pathways leading to decreased cellular proliferation, while integrin ligation reverses this signal and induces cell proliferation (Varner *et al.*, 1995). Recent findings have also shown that ligation of $\alpha_5\beta_1$ integrin can also promote cell survival by inhibiting apoptosis (Frisch and Francis, 1994; Varner *et al.*, 1995). Together, these data show that there is variability in $\alpha_5\beta_1$ expression on tumor cells and that its function in malignancy still remains unknown, but may depend on the tumor type and stage of progression.

We have developed a murine mammary carcinoma model, SP1, to study stromal cell interactions that affect tumor growth and metastasis. We have shown that adipocytes, a dominant cell type in mammary stroma, secrete hepatocyte growth factor (HGF) which is mitogenic for SP1 cells. Under anchorage-independent conditions SP1 cells grow as tight spheroids which contain an abundance of fibronectin fibrils, and only low amounts of other ECM proteins (Saulnier *et al.*, 1996). We therefore hypothesized that fibronectin and growth factors are required for growth of SP1 cells under anchorage-independent conditions. In this report, we examine the adhesive properties of SP1 cells and the effects of various extracellular matrix proteins and growth factors on anchorage-independent growth.

MATERIALS AND METHODS

Cell Lines

The SP1 tumour is a spontaneous non-metastatic murine mammary intraductal adenocarcinoma isolated in 1982 from an 18 month old CBA/J female retired breeder in the mouse colony at Queen's University. Subsequently, the SP1 carcinoma cell line was established, and characterized (Elliott *et al.*, 1992). The established SP1 cell line was frozen at -70°C to maintain stocks. Cells were kept in culture for no more than 3 months before thawing a fresh stock. All cell lines were tested for mycoplasma (Elliott *et al.*, 1992). In some experiments, clones of SP1 cells were isolated from single cells by limiting dilution.

Antibodies and Peptides

Rabbit antibody against the α_v integrin cytoplasmic domain was obtained from E. Ruoslahti (Burnham Institute, La

Jolla, CA). Rabbit anti-α_3 and anti-α_5 antibodies directed to the corresponding integrin cytoplasmic domain were a generous gift from R. Hynes (Howard Hughes Medical Institute, Cambridge, MA). Monoclonal antibody (GoH3) to the α_6 integrin subunit was obtained from A. Sonnenberg (Netherlands Cancer Institute, Amsterdam, Netherlands). A hybridoma cell line producing monoclonal anti-α_4 integrin antibody (R1-2) was obtained from American Tissue Type Culture collection (Rockville, MD). Monoclonal anti-α_2 integrin antibody (Fehlner-Gardiner et al., 1996a) and blocking anti-mouse α_5 integrin antibody (BMA5) (Fehlner-Gardiner et al., 1996b) were prepared as described previously. TGF-β was purchased from Sigma, Oakville, Ont., Canada). HGF was a gift from R. Schwall (Genentech, South San Francisco, CA). Rabbit anti-β_1 integrin antibody was obtained from K. Rubin (Biomedical Center, Univ. of Uppsala, Sweden). FN, laminin, collagen types I and IV and vitronectin were from Gibco BRL (Burlington, Ont.).

Colony Assay
Petri dishes (60 x 10 ml) were layered with 1.5 ml of a 1:1 mix of a 1.2% agar solution in ddH$_2$O and 2x RPMI (final concentration 0.6% agar and 1x RPMI) and the appropriate concentration of FBS. SP1 cells were then added (2500 cells/plate) in a 1:1 mixture of 0.72% agar and 2x RPMI (final concentration 0.36% and 1x RPMI). An aliquot (2.5 ml) of the mixture was added to each plate. After solidification, the plates were incubated at 37°C for 10 days. The colonies were fixed in 100% methanol, stained with Giemsa for 30 min. and counted manually.

Cell Surface Labelling and Immunoprecipitation
SP1 cells were grown in monolayers on tissue plastic or as colonies on agar-coated plates in RPMI 1640 supplemented with 7% FBS. The cells were harvested with 5 mM EDTA in PBS, washed once in PBS, resuspended in 1 ml and placed in a glass vial containing 100 µg iodogen (Pierce, Rockford, IL). The cells were surface labelled with 1.0 mCi ^{125}I for 20 min. at room temperature on a planar rotator. Unbound label was removed by washing 2 times with PBS. The cell pellets were lysed in RIPA lysis buffer containing 1.0 mM PMSF for 30 min. at 4°C. The cell lysates were spun to remove solid debris. Supernatants were precleared with rabbit anti-mouse IgG and protein A sepharose, aliquoted and incubated with the corresponding anti-integrin antibodies. Immunoprecipitates were washed 4 times in RIPA wash buffer, boiled for 3 min. in sample buffer and run on a 7.5% SDS-PAGE under non-reducing conditions.

Cl-12-H and Cl-24-L cells were labelled with a biotinylation reagent (40 µg/ml) (Amersham, Oakville, Ont., Canada) for 20 min. at room temperature to biotinylate all cell surface proteins. The cells were washed in PBS and lysed in RIPA lysis buffer. The integrins were immunoprecipitated with the corresponding antibodies and resolved by 7.0% SDS-PAGE. The proteins were transferred to nitrocellulose and a western blot performed using a Streptavidin-HRP conjugate for detection. The proteins were visualized using ECL reagents.

Cell Adhesion Assay
Linbro tissue culture plates (96 wells) were precoated with a two-fold serial dilution of ECM proteins starting at 20 µg/ml of FN, laminin, vitronectin, collagen type I, and collagen type IV at 4°C overnight. The following day the wells were washed with PBS, and blocked with 2.0 mg/ml BSA in RPMI for 2 hrs. at 37°C. Cells were harvested and resuspended in RPMI containing 0.5 mg/ml BSA. Cells (30,000/well) were plated in 100 µl and incubated at 37°C for 45 min. Unattached cells were removed by washing with PBS and the remaining cells were fixed in 3.7% paraformaldehyde and stained with 0.1% toluidine blue in a 1% NaBO$_4$ solution. The plates were washed gently with running water to remove excess dye and the absorbance measured at a wavelength of 570 nM in an ELISA plate reader.

Proliferation Assay
Cells (1 x 10^4) were plated in 24-well tissue culture plates in the test media. After 24 hrs., 0.2 µCi ^{3}H-thymidine (Amersham, Oakville Ont., Canada) were added to each well and incubated for another 24 hrs. at 37°C. The cells were harvested with trypsin-EDTA, placed in 96-well microtiter plates, then transferred to filters using a cell harvester (ICN Pharmceuticals, Costa Mesa, CA). ^{3}H-thymidine incorporation was measured (cpm/well) in a scintillation counter using Ecolume scintillation fluid.

RESULTS

TGF-β, HGF and Fibronectin stimulate Anchorage-independent Growth of SP1 Cells
Previously, we have shown that both HGF (Rahimi et al., 1994) and TGF-β (Rahimi et al., 1996) are secreted by adipocytes and that both HGF and TGF-β stimulate growth of SP1 colonies in agar (Saulnier et al., 1996). We now show that exogenous fibronectin added to SP1 agar colonies partially supports growth, but when added in combination with HGF or TGF-β shows little or no additive effect (Fig. 1). These results imply that fibronectin may be a contributing component in TGF-β- and HGF-induced colony growth.

Exogenous Fibronectin is required for Anchorage-independent Growth of SP1 Cells
The first step in investigating the role of extracellular matrix proteins, especially fibronectin, in colony growth was to remove the primary source of exogenous FN from the culture medium. The FBS was therefore subjected to immunoprecipitation with anti-bovine FN antibodies to remove FN.
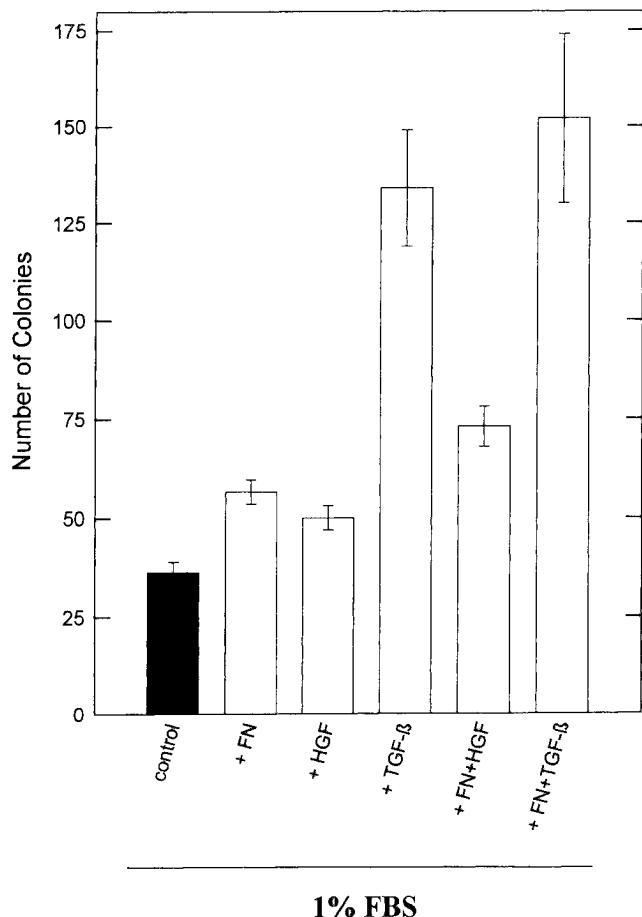
1% FBS

Fig. 1 *Effect of growth factors and fibronectin on anchorage-independent growth of SP1 cells.* The colony assay was performed in agar as described in the Materials and Methods. The control group contained 1% FBS/RPMI alone. For groups containing FN (10 μg/ml), HGF (5 ng/ml) and TGF-β (0.5 ng/ml), components were added at the concentrations indicated to the RPMI/agar mixture before solidification. The cultures were incubated for 14 days at 37°C, and colonies were fixed and stained as previously described (Saulnier *et al.*, 1996). The experiment was performed in quadruplicate. The results are expressed as the mean ± SD of quadruplicates. The experiment was repeated 2 times with similar results.

Fig. 2 *Effect of depleting fibronectin from serum on colony growth of SP1 cells.* Serum was either untreated (UT) or incubated with protein A sepharose (PAS) to remove endogenous IgG. After PAS treatment the serum was precleared with rabbit anti-mouse IgG and PAS (IgG). After preclearing the serum, FN was removed by immunoprecipitation with polyclonal rabbit anti-FN IgG (5 μl/ml serum) and PAS (anti-FN IgG). In one group bovine plasma FN was added to the culture medium at a concentration of 10 μg/ml (anti-FN IgG + FN). The colony assay was performed as described in the Materials and Methods in the presence of 7% FBS (untreated or treated as above). The cells were incubated for 14 days, then fixed in methanol and stained with Geimsa. The colonies were counted manually. The results are expressed as the mean colony number ± SD of quadruplicates. The results of four experiments showed a mean inhibition of 66.5% with a range of 26-89%, in the FN-reduced group compared to the 7% FBS control.

Fig. 2 demonstrates that immunoprecipitation of FN from serum reduced colony efficiency of SP1 cells, while endogenous IgG depletion with protein A sepharose alone, or preclearing with rabbit anti-mouse IgG, did not remove any factors required for growth in soft agar. Addition of exogenous bovine plasma FN to serum immunoprecipitated with anti-FN IgG, reconstituted more than half the colony growth. We also analysed the immunoprecipitates

of FN from serum on 6.0% SDS-PAGE under reduced conditions and demonstrated using silver staining that we were able to remove some FN from the serum (data not shown). These results show that

fibronectin plays an important role in SP1 colony growth.

Fibronectin promotes, and Collagen inhibits, Colony Growth of SP1 Cells

Having demonstrated that FN is required for colony growth in ideal culture conditions (7% FBS), we investigated whether addition of purified extracellular matrix to limiting culture conditions (1% FBS) will enhance colony growth. When SP1 cells are cultured in agar containing 1% FBS, very few colonies grow and colonies that do grow remain small in size. We used these limiting conditions to establish which ECM proteins affect anchorage-independent growth of SP1 cells. Addition of FN and laminin

marginally increased colony growth in 1% FBS, but these ECM proteins failed to generate large colonies as in 7 % FBS (Fig. 3). The increase in colony growth with fibronectin, although small, was reproducible in 4 of 4 experiments and the range of increase was from 29%-63% as compared to the 1% FBS control group. In contrast, both collagen types I and IV inhibited colony growth (by a range of 46%-96% in 4 experiments) in both 1% and 7% FBS (Fig. 3 inset). The concentration of collagen type I required to cause an inhibitory effect was 5 µg/ml or greater (data not shown). In addition, when collagen type I was combined with fibronectin there was still an inhibitory effect on colony growth. These data show that fibronectin alone is not suffi-
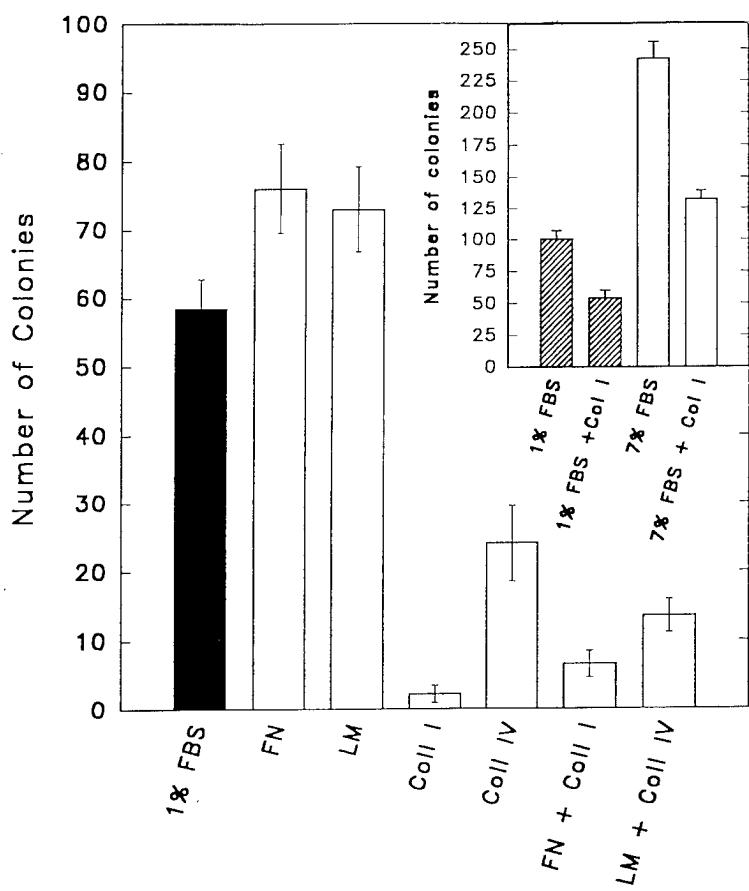
Fig. 3 *Effect of purified ECM proteins on SP1 colony growth.* The colony assay was performed as described in the Materials and Methods. The purified matrix proteins indicated in the X axis were added at a concentration of 10 µg/ml to the RPMI/agar mixture before solidification. All cultures contained 1% FBS. **Inset**) shows the effect of collagen type I on SP1 colonies in medium supplemented with 1% and 7% FBS. Colony growth was allowed to proceed for 14 days at 37°C. The colonies were fixed and stained as described in the Materials and Methods. The results are expressed as the mean ± SD of quadruplicates. In four experiments with FN an increase in colony growth ranging from 26% to 63%, compared to the 1% FBS control group was observed. In four experiments with collagen, a 67% mean inhibition of colony growth was observed ranging from 46%-96%, compared to the 1% FBS control group.

cient to promote optimal colony growth, and that collagen inhibits colony formation in a dominant manner.

SP1 Cells adhere to Fibronectin via $\alpha_5\beta_1$ Integrin

To determine whether other matrix proteins are likely to interact within the colonies, we determined the adhesive characteristics and integrin profile of SP1 cells. SP1 cells adhered and spread extensively to fibronectin and vitronectin over a wide range of concentrations (0.16-20 µg/ml). They adhered moderately well to collagen type I, and showed a concentration dependent requirement of approximately 1.5 µg/ml for adhesion to collagen type IV. SP1 cells attached poorly to laminin and few cells remained on BSA coated plates after washing with PBS (Fig. 4A). We next determined the integrin profile of SP1 cells under both adherent and anchorage-independent conditions, using cell surface labelling with [125]I. We pre-cleared the cell lysates with rabbit anti-mouse IgG before immunoprecipitation with the anti-integrin antibodies to help reduce non-specific binding to the IgG. Fig. 4B illustrates that the integrin expression under adherent and anchorage-independent conditions for 24 hrs. in the presence of 7% FBS is very similar. SP1 cells express a number of integrins on the cell surface including the FN receptor, $\alpha_5\beta_1$, the vitronectin receptor, $\alpha_v\beta_3$, the laminin receptor, $\alpha_6\beta_1$ and the collagen, laminin and fibronectin receptor, $\alpha_3\beta_1$. The collagen/laminin receptor, $\alpha_2\beta_1$, was weakly expressed on SP1 cells cultured on plastic but showed increased expres-

sion on SP1 cells maintained under anchorage-independent conditions for 24 hrs. (Fig. 4B). A similar gel was repeated 4 times with SP1 cells under adherent conditions or with a metastatic variant of SP1 cells. In the first two integrin profiles performed using ^{125}I-labelled cells, an IgG control was added and no non-specific bands were observed. Flow cytometry revealed very low expression of the FN receptor $\alpha_4\beta_1$ (data not shown). Adhesion to FN was almost completely blocked with anti-α_5 antibody, BMA5, a blocking antibody to the murine α_5 integrin subunit (Fig. 5). These data demonstrate that adhesion of SP1 cells to fibronectin occurs primarily via $\alpha_5\beta_1$ integrin, and that expression of the $\alpha_2\beta_1$ integrin is increased under anchorage-independent conditions.

An SP1 Cell Clone, Cl-12-H, grows efficiently in Agar in Serum-free Medium and expresses $\alpha_2\beta_1$

Since SP1 cells are clonally heterogenous, we isolated clones from SP1 cells and tested their ability to grow under anchorage-dependent and anchorage-independent conditions, and correlated the growth phenotype with cell adhesion and integrin expression profiles. From 35 clones isolated and tested, we selected two clones; Cl-12-H cells which form large colonies in agar and Cl-24-L cells which do not grow well in agar (Fig. 8A). The parent SP1 cells and the two clones had similar growth rates when cultured in 7% FBS on tissue culture plastic. However, in serum free conditions, the Cl-12-H clone grew much better than either SP1 or the Cl-24-L clone (Table 1). The adhesive

Table 1   *Proliferation of SP1 cells and clones Cl-12-H and Cl-24-L in two dimensional cultures*

	7% FBS	Serum-free
SP1	84,321 ± 26,593 cpm	14,467 ± 6,939 cpm
Cl-12-H	85,544 ± 9,589 cpm	27,638 ± 11,204 cpm*
Cl-24-L	87,847 ± 10,879 cpm	13,819 ± 8115 cpm

SP1, Cl-12-H and Cl-24-L cells were seeded (10,000/well) in 24 well tissue culture plates in the test medium (7% FBS or serum-free). After 24 hrs., 0.2 µCi ^{3}H-thymidine was added to each well and incubated for another 24 hrs. at 37°C. The cells were harvested with trypsin-EDTA and placed in 96-well microtiter plates then transferred to filters using a cell harvester. ^{3}H-thymidine incorporation was measured (cpm/well) in a scintillation counter using Ecolume scintillation fluid. The results are expressed as the mean ± SD of four experiments; each experiment contained quadruplicate samples. *indicates a significant increase in growth compared to SP1 and Cl-24-L cells grown under serum-free conditions ($p<0.05$).

Fig. 4 *Adhesion of SP1 cells to purified ECM proteins.* Panel **A**) The cell adhesion assay was carried out as described in the Materials and Methods. Linbro 96-well tissue culture plates were coated for 18 hrs. at 4°C with BSA, FN, laminin (LM), vitronectin (VN), collagen type I (Coll I) and collagen type IV (Coll IV) at concentrations of two-fold dilution ranging from 0.15 to 20 μg/ml. SP1 cells in RPMI 1640 supplemented with 0.5 mg/ml BSA were added to each well (30,000 cells /well) in a 100 μl vol. After 45 min. at 37°C, the unattached cells were removed by washing with warm PBS. The remaining cells were fixed and stained in a solution of 1% toluidine blue and 1% $NaBO_4$. The absorbance was measured with an ELISA plate reader at a wavelength of 570 nm. Each point is expressed as the mean ± SD of triplicates. The experiment was repeated three times with similar results. Panel **B**) SP1 cells were cultured on tissue culture plastic or as colonies on agar coated plates in RPMI 1640 medium supplemented with 7% FBS for 24 hrs. The cells were harvested and the number of cells normalized in each group, then surface labelled with 1.0 mCi ^{125}I. The cell pellets were lysed in RIPA buffer at 4°C for 30 min. and the supernatants were precleared with rabbit anti-mouse IgG and protein A sepharose. The supernatents were aliquoted and incubated with anti-integrin antibodies and protein A sepharose. Immunoprecipitates were washed 4 times in lysis buffer and run on a 7.5% SDS-PAGE under non-reducing conditions. Antibodies used were; from left to right, polyclonal anti-β_1; polyclonal anti-human-α_2 (cytoplasmic); polyclonal anti-human-α_3 (cytoplasmic); polyclonal anti-human-α_5 (cytoplasmic); mouse monoclonal anti-α_6 (GoH3) and polyclonal anti-human-α_v (cytoplasmic). The gel was fixed, dried and autoradiographed. A similar gel was repeated 4 times with similar results. "P"-cells grown on tissue culture plastic (adherent). "A"-cells grown on agar (non-adherent).

profiles of the Cl-12-H and Cl-24-L clones were very similar on fibronectin, laminin, vitronectin and osteopontin (data not shown). However the Cl-12-H clone adhered and spread on collagen, but the Cl-24-L clone did not (Fig. 6). The primary integrin receptors for collagen are $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$. Immunoprecipitation of the integrin receptors for collagen using anti-α_2, -α_3 and -β_1 antibodies sho-

Fig. 5 *Effect of blocking anti-α_5 antibody, BMA5, on adhesion of SP1 cells to fibronectin.* The cell adhesion assay was performed as described in Materials and Methods. Linbro 96 well tissue culture plates were precoated with fibronectin (10 µg/ml) or BSA (10 µg/ml) overnight at 4°C. SP1 cells were seeded (30,000 cells/well) in the presence of no antibody (NT), anti-α_5 (BMA5) or anti-α_6 (GoH3) antibodies (hybridoma conditioned media, 30% v/v), or rabbit anti-mouse IgG (30 µg/ml) (IgG). After 30 min. at 37°C the nonadherent cells were removed by washing with warm PBS. The remaining cells were fixed, stained and the absorbance measured in an ELISA plate reader at 570 nm. The results are expressed as the mean ± SD of triplicates. The experiment was repeated twice with similar results.

wed that Cl-12-H cells expressed higher levels of the $\alpha_2\beta_1$ integrin than Cl-24-L cells. Both cell lines expressed similar levels of the β_1 and the α_3 integrin (Fig. 7A). In the α_2 immunoprecipitation we observed a band at approximately 110 kDa which co-migrated with the β_1 subunit but disappeared upon reduction of the samples (Fig. 7B). We also observed a third band at approximately 100 kDa which is also present in the IgG control. This band was also lost after treatment with 2-mercaptoethanol. Both Cl-12-H and Cl-24-L cells expressed similar levels of the $\alpha_5\beta_1$ receptor (data not shown).

To determine whether adhesion to collagen contributes to the colony forming ability of Cl-12-H cells, we compared the ability of fibronectin and collagen to promote or inhibit colony growth of the two clones. Collagen type I inhibited colony growth of SP1 cells in 7% FBS medium, promoted Cl-12-H colony growth in agar, and had no effect on colony growth of Cl-24-L cells (Fig. 8B). Fibronectin also promoted colony growth of Cl-12-H cells, but had no effect on Cl-24-L cells (Fig. 8B). These results suggest a role for the $\alpha_2\beta_1$ receptor in anchorage-independent growth of SP1 cells.

DISCUSSION

The role of extracellular matrix in cell adhesion, growth and differentiation has been well demonstrated in 2-dimensional cultures. However the role of ECM in 3-dimensional spheroids is not well understood. It has been shown that tumor spheroids contain ECM proteins *in vitro* (Nederman *et al.*, 1984) and that ECM proteins such as fibronectin can enhance lung colonization of B16 melanoma cells in lungs (Coucke *et al.*, 1992). These results suggest a role for ECM proteins in anchorage-independent growth, however no direct correlation has been demonstrated between the presence of ECM and anchorage-independent growth potential. Our objective in this report was to demonstrate that soluble ECM proteins, in this case, fibronectin and collagen, can interact with cells under anchorage-independent conditions and can modulate colony
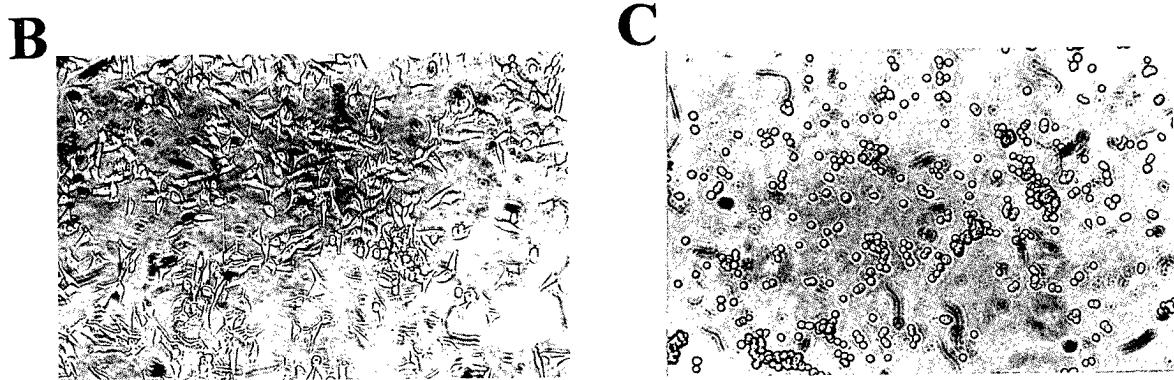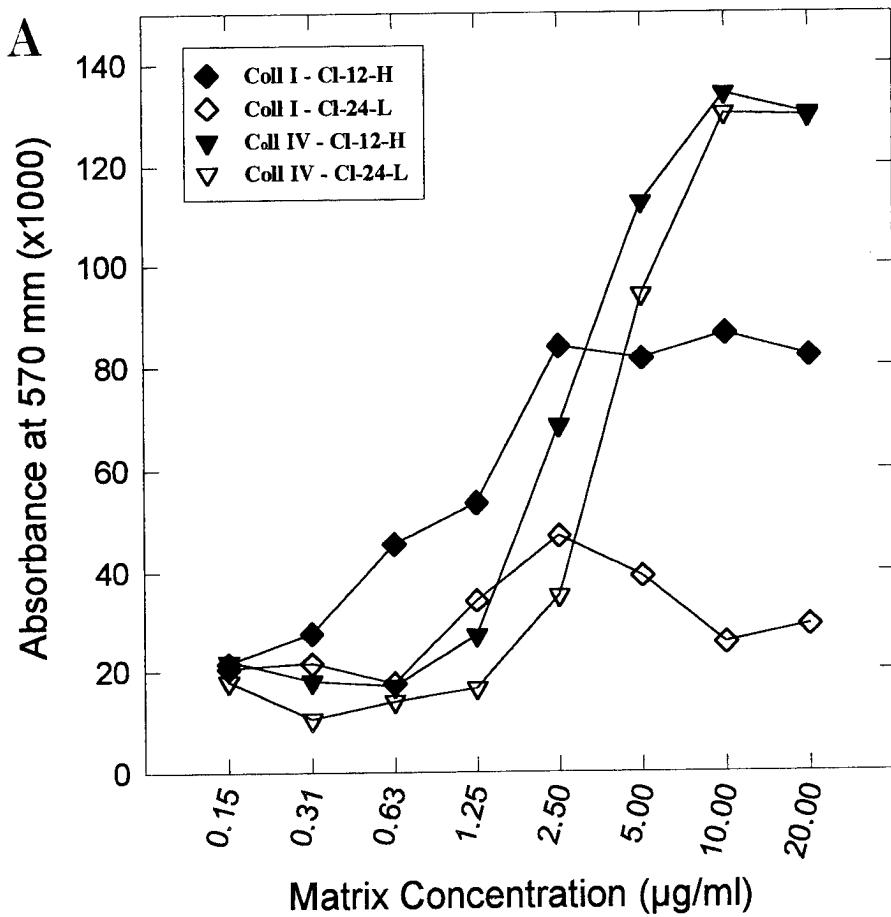
Fig. 6   *Comparison of collagen types I and IV binding properties of Cl-12-H clone and Cl-24-L clone.* Panel A) The cell adhesion assay was performed as described in Materials and Methods, with plates coated with collagen types I or IV at the indicated concentrations. Panels B) and C) Morphology of Cl-12-H and Cl-24-L cells after plating for 45 min. on dishes coated with collagen type I is shown. Non-adherent cells were not removed.
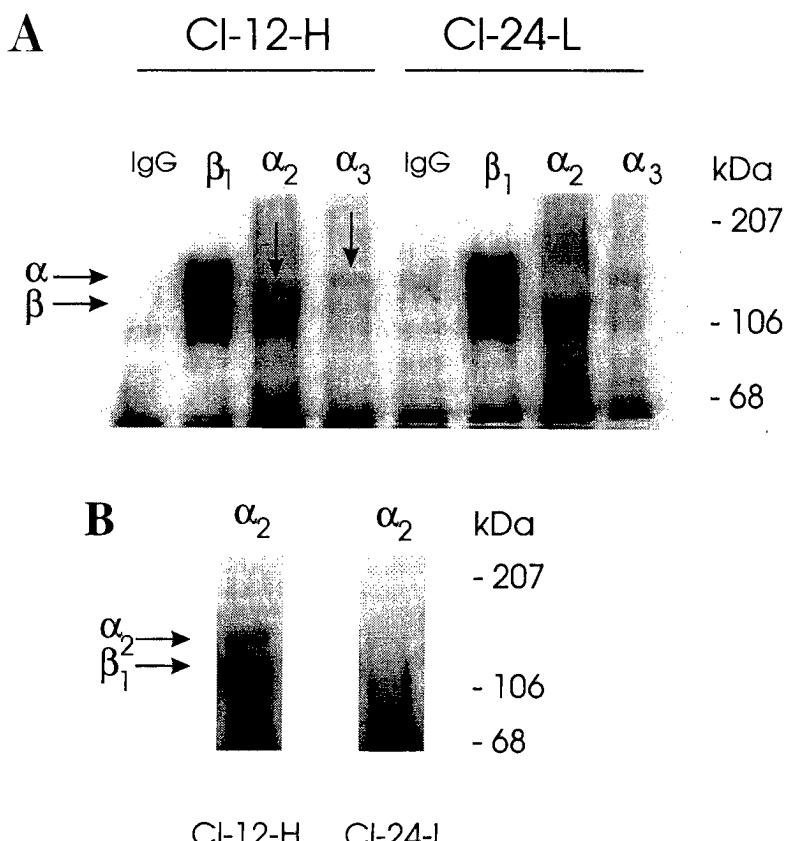
Fig. 7 *Comparison of integrin profile of Cl-12-H clone and Cl-24-L clone.* Panel **A**) Cl-12-H and Cl-24-L cells were harvested and labelled with a biotinylation reagent to biotinylate all cell surface proteins as described in the Materials and Methods. The cells were counted and the cell number normalized in each group then lysed in RIPA lysis buffer. The lysates were centrifuged and the protein concentration of the supernatant normalized and aliquoted into 4 groups. The lysates were immunoprecipitated with rabbit anti-mouse IgG (control), rabbit anti-β_1, anti-α_2 and anti-α_3 IgG and resolved by 7.5% SDS-PAGE under non-reducing conditions. The proteins were transferred to nitrocellulose and a western blot analysis was performed using a streptavidin-horse radish peroxidase-conjugated antibody for detection. The proteins were visualized using ECL reagents. The vertical arrows ↓ show the position of the α_2 and α_3 subunits of Cl-12-H cells. Panel **B**) An aliquot of the same immunoprecipitate as in Panel A was run under reduced conditions (5% 2-mercaptoethanol) and resolved by 7.0% SDS-PAGE. The proteins were transferred to nitrocellulose and a Western blot analysis performed using a streptavidin-horse radish peroxidase-conjugated antibody for detection. The proteins were visualized using ECL reagents.

growth. SP1 cells can survive and grow well anchorage-independently in the presence 7% FBS. By reducing the serum concentration to 1%, we have created conditions which limit growth in agar. Under these conditions, we can easily test what ECM proteins or growth factors positively or negatively affect anchorage-independent growth.

We have previously demonstrated that FN secretion from SP1 cells is dependent on cell spreading (Saulnier *et al.*, 1996). SP1 cells grown in suspen-

sion showed reduced FN synthesis and secretion within 5 hrs., suggesting that endogenous FN becomes limiting for growth of SP1 cells under anchorage-independent conditions. These results, along with others presented here, suggest that an exogenous source of fibronectin is required for SP1 colony growth. However, our results have further demonstrated that fibronectin is not sufficient for anchorage-independent growth of SP1 cells, since addition of FN alone to 1% FBS cultures only marginally increased colony growth. Growth factors
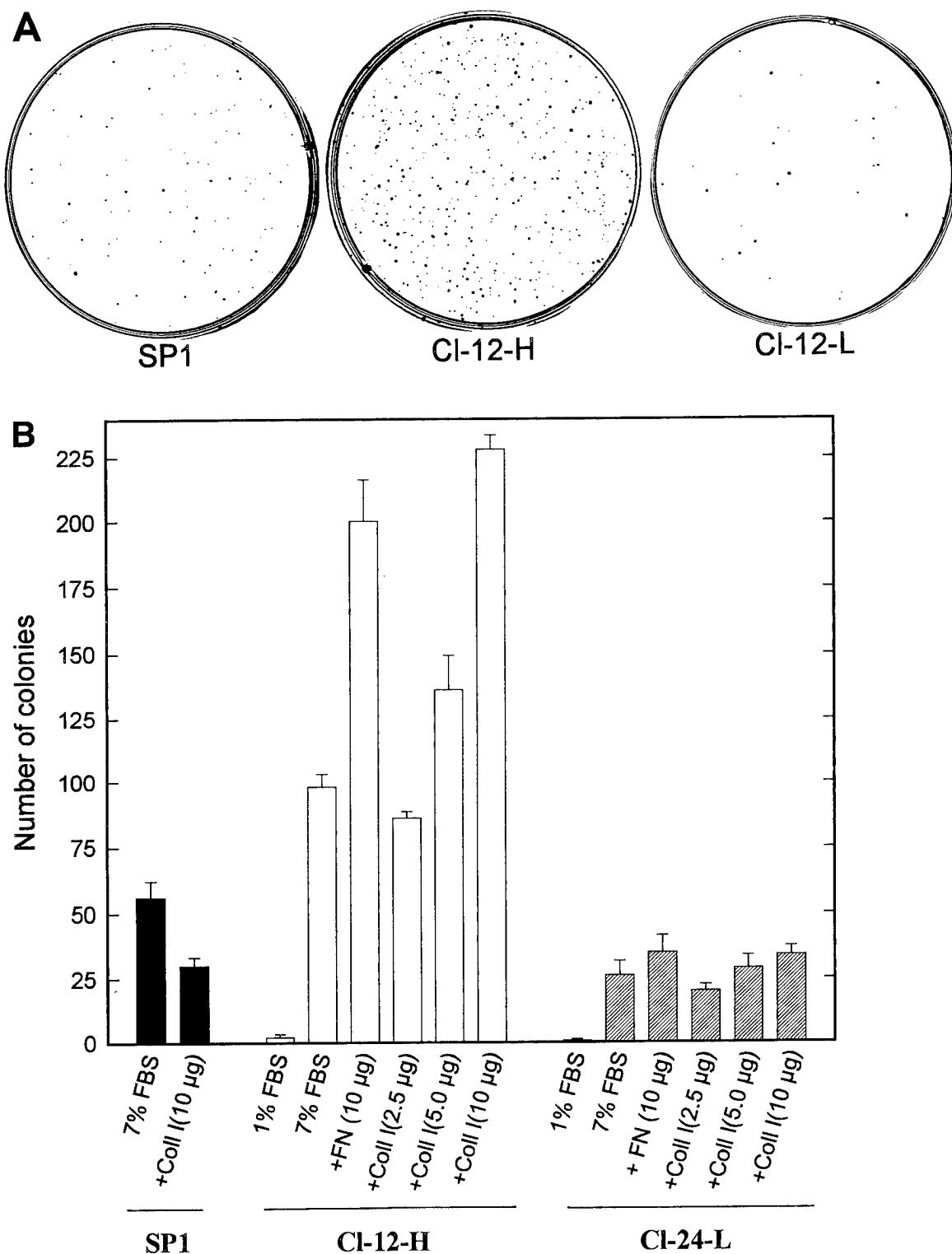
Fig. 8 *Effect of fibronectin and collagen on SP1, Cl-12-H and Cl-24-L colony growth.* The colony assay was performed as described in the Materials and Methods. Panel **A**) The growth morphology of Cl-12-H and Cl-24-L colonies in agar supplemented with 7% FBS is compared. Panel **B**) Fibronectin or collagen type I was added in the agar at the concentrations (μg/ml) indicated in parentheses. The experiment was performed in quadruplicate and the mean ± SD of each group was calculated.

(HGF and TGF-β) and other undefined components are also required for optimal colony growth. We are currently investigating whether TGF-β and HGF, which promote SP1 colony growth, induce synthesis of FN and $\alpha_5\beta_1$ and thereby regulate fibronectin adhesion under anchorage-independent conditions.

In contrast to the results obtained with FN, collagen inhibits SP1 colony growth. The inhibitory effect of collagen on SP1 anchorage-independent growth is either indirect (i.e. due to interference with binding to fibronectin which generates a positive growth signal), or direct (i.e. due to binding of collagen to specific integrin receptors which generate a negative growth signal). Since SP1 cells express few receptors for collagen and the inhibition of growth maintains a linear relationship with increasing concentrations, we speculate that the mechanism is indirect but further investigation is required to confirm this hypothesis. Increased adhesiveness to collagen via the $\alpha_2\beta_1$ receptor may play a role in colony growth. In support of this possibility, we have isolated a clone (CL-12-H) of SP1 cells which grows efficiently in an anchorage-independent manner, expresses higher surface expression of the collagen receptor $\alpha_2\beta_1$, and grows in response to collagen under anchorage-independent conditions. In contrast, the parent SP1 cells under anchorage-independent conditions express more $\alpha_2\beta_1$ than adherent cells (Fig. 4), but show inhibition of growth in response to collagen. This apparent discrepancy may be explained by selection processes. SP1 cells are a heterogenous population of cells, some of which express the $\alpha_2\beta_1$ receptor, and others which do not. When SP1 cells are cultured in soft agar in the presence of collagen there is a net reduction in the number of colonies, however, a few colonies grow well under these conditions. Cl-12-H cells are most likely a clone of this cell phenotype. These results also suggest that a significant level of $\alpha_2\beta_1$ expression may be required on the cell surface in order to obtain a positive growth signal.

The observation that Cl-24-L cells do not form colonies in the presence of fibronectin suggests that other components in addition to ECM-integrin interactions are important for permitting anchorage-

independent growth. For example, autocrine production of growth factors (e.g. HGF or TGF-β) or activation of certain oncogenes may be required.

In addition to cooperating with growth factors in promoting cell growth, adhesion to fibronectin has been shown to facilitate cell survival by inhibiting apoptosis (Frish *et al.*, 1994; Varner *et al.*, 1995). In these systems, the apoptopic inhibitor Bcl-2 is downregulated upon loss of adhesion resulting in increased activation of interleukin-1β converting enzyme (Boudreau *et al.*, 1995). We have found that fibronectin can promote survival of SP1 cells under anchorage-independent conditions and are investigating the role of Bcl-2 in integrin-dependent cell survival of SP1 cells (data unpublished).

In summary, our findings in this report and elsewhere (Saulnier *et al.*, 1996) show that both growth factors and ECM proteins affect growth and survival of SP1 carcinoma cells under anchorage-independent conditions. These observations support the concept that loss of the anchorage-dependency requirement in malignancy is a stepwise process (Fig. 9). SP1 cells represent an intermediate stage in which integrin ligation and activation of growth factor receptors are still required, however cell spreading is not. Similarly, direct ligation of the $\alpha_5\beta_1$ integrin receptor with anti-α_5 antibody was shown to stimulate growth and survival of human melanoma cells (Mortarini *et al.*, 1992) and RGD-containing peptides were shown to stimulate anchorage-independent growth of K562 cells (Symington, 1995). Over-expression of cyclin A, required for G1-S phase transition (Guadagno *et al.*, 1993), or of Bcl-2 which inhibits apoptosis (Frisch *et al.*, 1995), are two possible mechanisms by which integrin ligation might affect cell growth. Further studies are underway to better understand the mechanisms which regulate anchorage-independent growth and the contribution of ECM and growth factors.

Stepwise progression for colony formation
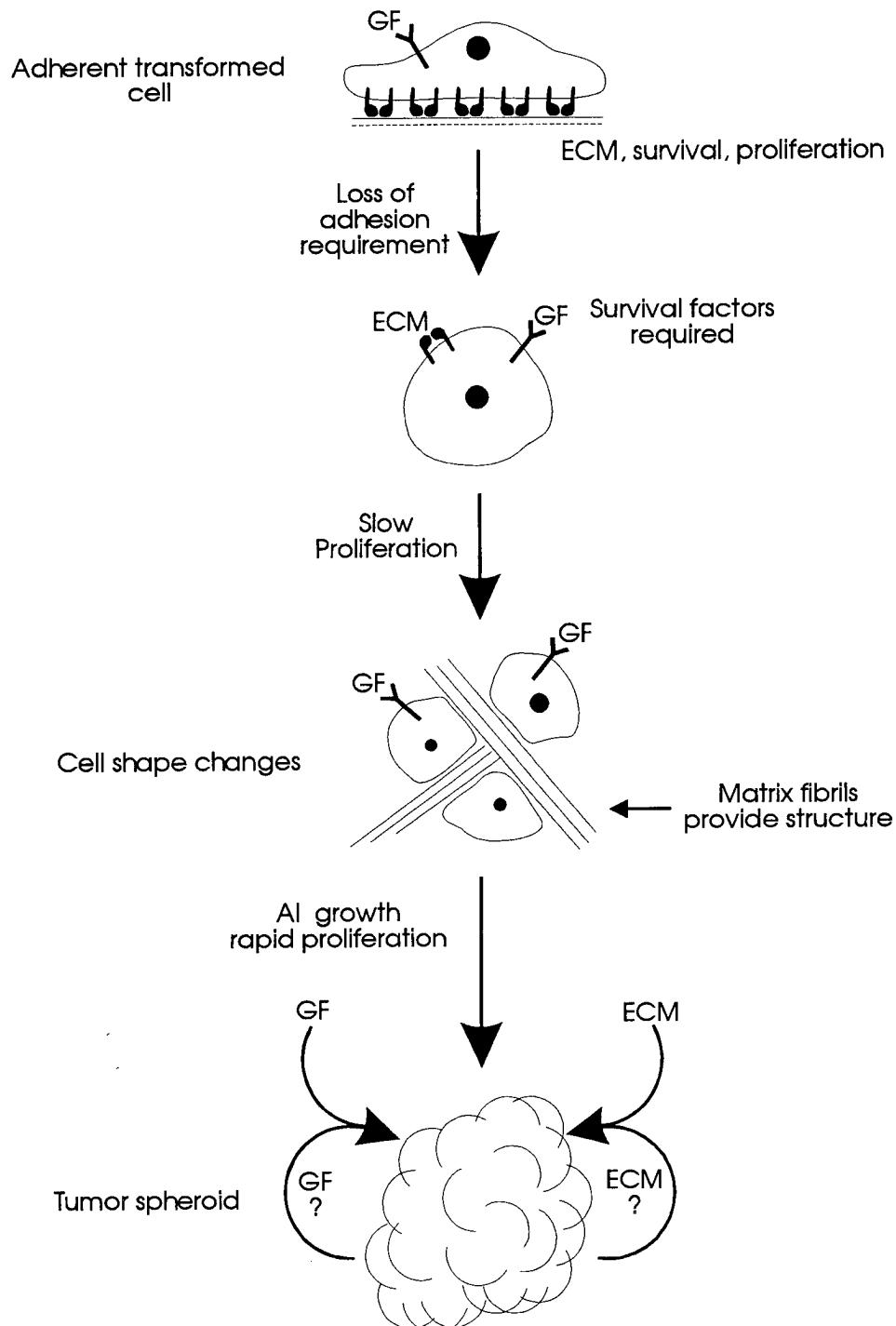
Fig. 9 *Proposed model for stepwise progression of colony formation.* Based on our results, we propose the following model. Adherent transformed cells progressively lose the requirement for adhesion. Once in an anchorage-independent state, soluble extracellular matrix proteins (ECM) bind to integrin receptors (⌐), and growth factors (GF) bind to their respective receptors (Y) on the cell surface. These interactions provide survival signals to the cell. The cell then can slowly start to proliferate and organize ECM on its cell surface. Once several cells have clustered together, there is formation of ECM fibrils and a change in cell shape which provides the adhesive signals required to proliferate more rapidly and form a three-dimensional spheroid.

REFERENCES

Akiyama, S.K., Yamada, S.S., Chen, W.T. and Yamada, K.M., Analysis of fibronectin receptor function with monoclonal antibodies:role in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *J. Cell. Biol.* 1989, **109**: 863-875.

Albelda, S.M. and Buck, C.A., Integrins and other cell adhesion molecules. *FASEB J.* 1990, **4**: 2868-2880.

D'Ardenne, A.J., Richman, P.I., Horton, M.A., McAulay, A.E. and Barnard, N.J., Co-ordinate expression of the alpha-6 integrin laminin receptor subunit and laminin in breast cancer. *J. Pathol.* 1991, **165**: 213-220.

Boudreau, N., Sympson, C.J., Werb, Z. and Bissell, M.J., Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science* 1995, **267**: 891-893.

Coucke, P., de Pauw-Gillet, M.C., de Leval, L., Noel, A., Bassleer, R. and Foidart, J.M., Fibronectin promotes lung colony formation in the mouse by B16 melanoma cell spheroids. *In Vivo* 1992, **6**: 481-486.

Elliott, B., Tam, S.P., Dexter, D. and Chen, Z.Q., Capacity of adipose tissue to promote growth and metastasis of a murine mammary carcinoma: Effect of estrogen and progesterone. *Cancer Res.* 1992, **51**: 416-424.

Fehlner-Gardiner, C., Uniyal, S. and Chan, B.M.C., VLA-6 ($\alpha_6\beta_1$) integrin is transiently expressed during the development of mouse bone marrow-derived mast cells. *Develop. Growth Diff.* 1997, in press.

Fehlner-Gardiner, C., Uniyal, S., von Ballestrem, C. and Chan, B.M.C., Differential utilization of VLA-4 ($\alpha_4\beta_1$) and -5 ($\alpha_5\beta_1$) integrins during the development of mouse bone marrow-derived mast cells. *Differentiation* 1996, **60**: 317-325.

Folkman, J. and Moscona, A., Role of cell shape in growth control. *Nature* 1978, **273**: 345-349.

Freedman, V.H. and Shin, S., Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell* 1974, **3**: 355-359.

Frisch, S.M. and Francis, H., Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* 1994, **124**: 619-626.

Giancotti, F.G. and Mainiero, F., Integrin-mediated adhesion and signaling in tumorigenesis. *Biochim. biophys. Acta* 1994, **1198**: 47-64.

Giancotti, F.G. and Ruoslahti, E., Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell* 1990, **60**: 849-859.

Glimelius, B., Norling, B., Nederman, T. and Carlsson, J., Extracellular matices in multicellular spheroids of human glioma origin: Increased incorporation of proteoglycans and fibronectin as compared to monolayer cultures. *APMIS* 1988, **96**: 433-444.

Guadagno, T.M., Ohtsubo, M., Roberts, J.M. and Assoian, R.K., A link between cyclin A expression and adhesion-dependent cell cycle progression. *Science* 1993, **262**: 1572-1575.

Hangan, D., Uniyal, S., Morris, V., MacDonald, I., von Ballestrem, C., Chau, T., Schmidt, E., Chambers, A.,

Groom, A. and Chan, B., Integrin VLA-2 ($\alpha_2\beta_1$) function in postextravasation movement of human Rhabdomyosarcoma RD cells in the liver. *Cancer Res.* 1996, **56**: 3142-3149.

Hart, I.R. and Saini, A., Biology of tumour metastasis. *Lancet* 1992, **339**: 1453-1457.

Hynes, R.O., Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 1992, **69**: 11-25.

Miettinen, M., Castello, R., Wayner, E. and Schwarting, R., Distribution of VLA integrins in solid tumors. Emergence of tumor-type-related expression patterns in carcinomas and sarcomas. *Am. J. Pathol.* 1993, **142**: 1009-1018.

Mortarini, R., Gismondi, A., Santoni, A., Parmiani, G. and Anichini, A., Role of the $\alpha_5\beta_1$ integrin receptor in the proliferative response of quiescent human melanoma cells to fibronectin. *Cancer Res.* 1992, **52**: 4499-4506.

Nederman, T., Norling, B., Glimelius, B., Carlsson, J. and Brunk, U., Demonstration of an extracellular matrix in multicellular tumor spheroids. *Cancer Res.* 1984, **44**: 3090-3097.

De Pauw-Gillet, M.C., Christiane, Y.M., Foidart, J.M. and Bassleer, R.J.B., Analysis of tridimentional mixed cultures of mouse B16 melanoma cells and 3T3 fibroblasts. *Anticancer Res.* 1988, **8**: 153-160.

Peña, L., Nieto, A., Perez Alenza, M.D., Rodriguez, A., Sanchez, M.A. and Casteno, M., Expression of fibronectin and its integrin receptor $\alpha_5\beta_1$ in canine mammary tumours. *Res. Vet. Sci.* 1994, **57**: 358-364.

Pignatelli, M., Hanby, A.M. and Stamp, G.W.H., Low expression of $\beta 1$, α_2 and α_3 subunits of VLA integrins in malignant mammary tumours. *J. Pathol.* 1991, **165**: 25-32.

Poste, G. and Fidler, I.J., The pathogenesis of cancer metastasis. *Nature* 1980, **283**: 139-145.

Rahimi, N., Saulnier, R., Nakamura, T., Park, M. and Elliott, B.E., Role of hepatocyte growth factor in breast cancer: a novel mitogenic factor secreted by adipocytes. *DNA Cell Biol.* 1994, **13**: 1189-1197.

Saulnier, R., Bhardwaj, B., Klassen, J., Leopold, D., Rahimi, N., Tremblay, E., Mosher, D. and Elliott, B.E., Fibronectin fibrils and growth factors stimulate anchorage-independent growth of a murine mammary carcinoma. *Exp. Cell Res.* 1996, **222**: 360-369.

Symington, B.E., Growth factor signalling through the alpha5beta1 fibronectin receptor *Biochem. biophys. Res. Commun.* 1995, **208**: 126-134.

Varner, J.A., Emerson, D.A. and Juliano, R.L., Integrin $\alpha_5\beta_1$ expression negatively regulates cell growth: reversal by attachment to fibronectin. *Mol. Biol. Cell* 1995, **6**: 725-740.

Wu, C., Bauer, J.S., Juliano, R.L. and McDonald, J.A., The $\alpha_5\beta_1$ integrin fibronectin receptor, but not the α_5 cytoplasmic domain, functions in an early and essential step in fibronectin matrix assembly. *J. biol. Chem.* 1993, **268**: 21883-21888.

Zutter, M.M., Santoro, S,A., Staatz, W. and Tsung, Y.L., Re-expression of the $\alpha_2\beta_1$ integrin abrogates the malignant phenotype of breast carcinoma cells. *PNAS* 1995, **92**: 7411-7415.

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